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title = NOVEL METHODS FOR THERAPEUTIC VACCINATION

FIELD OF THE INVENTION

The present invention relates to novel methods for combatting diseases, such as cancers, which are characterized by the presence of cell-associated gene expression products which are non-immunogenic or poorly immunogenic. In particular, the present invention relates to methods for inducing an immune response conducted by cytotoxic T-lymphocytes (CTLs), whereby cells carrying epitopes from the gene expression products are attacked and killed by the CTLs. The invention also relates to a method of preparing immunogenic, modified polypeptide antigens which are derived from weakly immunogenic antigens.

The invention further relates to a series of applications of Applicant's AutoVac technology (which is the subject of WO 95/05849) within the field of therapeutic vaccination against cancer.

BACKGROUND OF THE INVENTION

The idea of vaccinating against cancer has been around for more than hundred years and has enjoyed recurrent bursts of activity, particularly since the turn of this century.

However, during the past 10 years the understanding of the fundamental molecular mechanisms of the immune response has improved considerably. Among the most important milestones achieved during this period has been the discovery of the still growing list of cytokines and growth factors, the understanding of the mechanisms of interaction between T and B cells as well as the establishment of the cellular antigen processing pathways including the role and structure of the MHC class I and II molecules in antigen presentation. Important discoveries with regard to cancer immunology - although still not fully understood - were also the

elucidation of the mechanisms underlying induction of immunological tolerance in a host. All this research has led to a huge amount of efforts in order to develop new treatments for human cancer.

Depending on how tumour immunity is acquired by the patient, immunotherapy regimens can be categorised as either passive or active. In passive immunotherapy regimens the patient passively receives immune components such as cytokines, antibodies, cytotoxic T-cells, or lymphocyte activated killer (LAK) cells. In contrast, active specific immunotherapy protocols encompass actively inducing tumour immunity by vaccination with the tumour cell or its antigenic components. This latter form of treatment is preferred because the immunity is prolonged.

Passive and active cancer vaccines have focussed on inducing either humoral or cellular immune responses. For active vaccines it is well established that induction of CD4 positive T helper cells is necessary in order to secondarily induce either antibodies or cytotoxic CD8 positive T cells.

Passive vaccination with antibodies

Since the discovery of the monoclonal antibody technology in the mid-seventies, a large number of therapeutic monoclonal antibodies directed against tumour specific or tumour associated antigens has been developed. Monoclonal antibody therapy, however, gives rise to several serious problems:

- Injection of these foreign substances induces an immune response in the patient towards the injected antibodies, which may lead to less efficient treatment as well as to serious allergic side-effects in the patients.

- Monoclonal antibodies usually must be administered in large amounts. This is a problem, since the production costs of monoclonal antibodies are huge.

- Monoclonal antibodies must be administered via the parenteral route and due to the relatively large amounts needed, the patients frequently must be hospitalised during the treatment.

- Injections of monoclonal antibodies must be repeated at rather short intervals (weeks) in order to maintain therapeutic effect.

- Monoclonal antibodies are usually not able to activate secondary effector systems of the immune system such as complement, NK-cells or macrophage killing of tumour cells.

The latter disadvantage is of particular importance in cancer therapy and may be an important reason why monoclonal antibody therapy of cancer in several cases has not been particularly successful. The so-called humanised monoclonal antibodies now used by many companies are less immunogenic, but unfortunately they are even less capable of activating the secondary immune effector systems. Furthermore, examples of secondary out-growth of tumours lacking the original tumour antigen have been observed, since these antibodies do not induce "innocent bystander" effects on tumour cells not carrying the tumour antigen.

The poor effector capability of the monoclonal antibodies has led to the development of monoclonal antibodies chemically conjugated to different toxins and radioisotopes. Pharmacia Upjohn AB has e.g. developed a conjugate between a monoclonal tumour specific antibody and the *Staphylococcus aureus* toxin A

with the purpose of activating T cells in the tumour. Medarex Inc. has developed bispecific monoclonal antibodies containing a tumour specific Fab fragment as well as an Fc-receptor specific antibody fragment with the purpose of activating macrophage killing of tumour cells. Both constructs are more effective than the monoclonal antibody alone, but they are also more expensive and immunogenic. Antibodies conjugated to radioisotopes are also expensive as well as immunogenic and other general toxic side-effects are observed.

The appearance of the monoclonal antibody technology was a major step forward which enabled the production of well-defined, high-affinity binding molecules. However, being monoclonal these antibodies only react with a single type of epitope on a tumour antigen. This is the major reason why they usually are not able to activate the complement system or binding to the Fc-receptors of NK-cells and macrophages. These very powerful effector systems usually require the co-localisation of multiple Fc antibody fragments protruding from the antigen.

Other researchers have therefore attempted to use two monoclonal antibodies in combination and this has led to an improved effect. It therefore seems very reasonable instead to attack tumour cells with highly specific polyclonal antibodies directed against a tumour specific, or against (over-expressed) tumour associated antigens or growth factor receptors. Such antibodies would be fully capable of activating the secondary effector systems mentioned above. Furthermore, it is likely that the local inflammatory reaction induced by these effector systems could lead to secondary effects on "innocent bystander" cells not expressing the tumour antigen in question as well as to activation of tumour specific TIL's (tumour infiltrating lymphocytes) in the tumour

tissue. Such effects have been observed by Medarex Inc. using their bi-specific monoclonal antibody conjugates.

Since the discovery of the monoclonal antibody technology the potential use of polyclonal antibodies for cancer therapy has not been explored very much (except for the antigens described below). One major reason is that well-defined tumour specific or tumour associated surface antigens only have been characterised within the recent years, but - more importantly - many of these have turned out to be self-antigens and therefore non-immunogenic. Accordingly, xenogenic polyclonal antibodies would necessarily have been used to study the effects. However, such antibodies induce a vigorous immune response towards the injected foreign polyclonal antibodies which rapidly eliminate the therapeutic effects.

Active vaccination to induce antibodies

Recent attempts to induce therapeutic polyclonal autoantibodies in cancer patients by active vaccination have been successful. Vaccines against membrane bound carbohydrate self-antigens (such as the O-linked aberrantly expressed Tn and sTn-antigens and the ganglioside liposaccharides GM2 and GD3) have been developed. These small carbohydrate structures are, however, very poor antigens so conjugates of these molecules with carrier molecules such as keyhole limpet haemocyanin (KLH) or sheep mucins (containing Tn- and sTn) must be used. In melanoma patients the induction of anti-GM2 antibodies were associated with a prolonged disease-free interval and overall survival after a minimum follow-up of fifty-one months. Also randomised phase II studies have been conducted on breast cancer patients using a conjugate of sTn and KLH in the DETOX-B adjuvant (BIOMIRA Inc.) showing that sTn immune patients had a significantly longer median survival compared to controls. Another example of the active induction

of polyclonal antibodies in cancer is the use of idiotype specific vaccination against B-cell lymphomas, which - although it has been promising - is limited to this cancer type only.

Finally, the US company Aphton Inc. has developed active conjugate vaccines against gonadotropin releasing hormone (GnRH) and gastrin. It has been demonstrated, that this vaccine is capable of controlling the biological activity of these hormones, which also can function as autocrine growth factors for certain tumour cells. Successful phase II clinical trials have been conducted on gastrointestinal cancer patients and phase III clinical trials are underway.

Cytotoxic T-cells

It has been clearly demonstrated by several groups that tumour specific cytotoxic T cells (CTL´s) are present in many tumours. These CTL's are termed tumour infiltrating lymphocytes (TIL's). However, these cells are somehow rendered non-responsive or anergic by several different possible mechanisms including secretion of immunosuppressive cytokines by the tumour cells, lack of co-stimulatory signals, down regulation of MHC class I molecules etc.

There has been many attempts to isolate the tumour specific HLA class I bound peptides recognised by TILs, and in some cases it has also been successful (e.g. peptides from the melanoma associated antigens). Such peptides have been used to induce a tumour specific immune response in the host, but the practical use of tumour specific peptides in vaccines is restricted to a limited segment of the population due to the narrow HLA class I binding specificity of the peptides. Furthermore, it is usually relatively difficult to evoke a CTL response *in vivo* using synthetic peptides due to the low

biological half-life of these substances as well as the difficulties with exogenous priming of MHC class I molecules.

Many other approaches have been attempted in order to evoke a tumour specific CTL response including the use of cytokines (e.g. IL-2, IFN-γ, IL-6, IL-4, IL-10 or GM-CSF) or co-stimulatory molecules (B7) either in soluble form or expressed by the transfected tumour cell. Furthermore, immunisations with allogenic or autologous whole cells, or of tumour antigens prepared in specialised adjuvants designed to present the antigen via the MHC class I antigen presentation route, or tumour antigens expressed in e.g. vaccinia vectors etc. have been used with varying success. Still the general belief among tumour immunologists is therefore that one of the best ways to eliminate tumours would be to induce a strong specific anti-tumour CTL response.

Apart from the fact that these treatments usually are very expensive and difficult to reproduce, it has also turned out to be difficult to obtain a good immune response towards the tumour since many of the tumour associated antigens are true self-proteins to which most T cells appear to be tolerant. Therefore, it seems necessary to induce a controlled cellular autoimmune condition in the patient.

OBJECT OF THE INVENTION

It is an object of the present invention to provide improved methods and agents for inducing immune responses in host organisms against undesirable antigens, e.g. tumour antigens. It is a further object to provide a method for preparing polypeptide analogues of such undesirable antigens, analogues which are capable of inducing an effective immune response against the undesired antigen.

SUMMARY OF THE INVENTION

Presentation of antigens has dogmatically been thought of as two discrete pathways, a class II exogenous and a class I endogenous pathway.

Briefly, a foreign protein from outside the cell or from the cell membrane is taken up by the APC as an endosome which fuses with an intracellular compartment which contains proteolytic enzymes and MHC class II molecules. Some of the produced peptides bind to class II, which then are translocated to the cell membrane.

The class I endogenous pathway is characterised by the predominant presentation of cytosolic proteins. This is believed to occur by proteasome mediated cleavage followed by transportation of the peptides into the endoplasmic reticulum (ER) via TAP molecules located in the membrane of the ER. In ER the peptides bind to class I followed by transportation to the plasma membrane.

However, these 2 pathways are not fully distinct. For example it is known that dendritic cells and to some extend macrophages are capable of endocytosing (pinocytosing) extracellular proteins and subsequently present them in the context of MHC class I. It has also previously been demonstrated that using specialised administration routes, e.g. by coupling to iron oxide beads, exogenous antigens are capable of entering the Class I pathway (Rock, 1996). This mechanism seems central, because of the importance of a concomitant expression of both class I and class II on the same APC to elicit a three cell type cluster. This three cell type cluster of interaction has been proposed by Mitchison (1987) and later by other authors. They showed the importance

of concomitant presentation of class I and class II epitopes on the same APC.

It has previously been demonstrated that insertion of a foreign MHC class II restricted T helper cell epitope into a self-antigen results in the provision of an antigen capable of inducing strong cross-reactive antibody responses directed against the non-modified self-antigen (cf. applicant's WO 95/05849). It was shown that the autoantibody induction is caused by specific T cell help induced by the inserted foreign epitope.

However, we have come to the conclusion that modified self-antigens - with the aid of appropriate adjuvants - ought to be capable of also inducing strong CTL responses against MHC class I restricted self-epitopes and hence the technology described in WO 95/05849 can be adapted to also provide vaccination against intracellular and other cell-associated antigens which have epitopes presented in the context of MHC Class I.

The autovaccine technology described in WO 95/05849 has the effect that specific T cell help is provided to self-reactive B cells when a modified self-antigen is administered for uptake into the MHC class II antigen processing pathway (cf. Fig. 1, and Dalum I et al., 1996, J. Immunol. 157: 4796-4804). It was shown that potentially self-reactive B-lymphocytes recognizing self-proteins are physiologically present in normal individuals. However, in order for these B-lymphocytes to be induced to actually produce antibodies reactive with the relevant self-proteins, assistance is needed from cytokine producing T-helper lymphocytes (T_H-cells or T_H-lymphocytes). Normally this help is not provided because T-lymphocytes in general do not recognize T-cell epitopes derived from self-proteins when presented by antigen presenting cells (APCs).

However, by providing an element of "foreignness" in a self-protein (*i.e.* by introducing an immunologically significant modification), T-cells recognizing the foreign element are activated upon recognizing the foreign epitope on an APC (such as, initially, a mononuclear cell). Polyclonal B-lymphocytes (which present T-cell epitopes) capable of recognising self-epitopes on the modified self-protein also internalise the antigen and subsequently presents the foreign T-cell epitope(s) thereof, and the activated T-lymphocytes subsequently provide cytokine help to these self-reactive polyclonal B-lymphocytes. Since the antibodies produced by these polyclonal B-lymphocytes are reactive with different epitopes on the modified polypeptide, including those which are also present in the native polypeptide, an antibody cross-reactive with the non-modified self-protein is induced. In conclusion, the T-lymphocytes can be led to act as if the population of polyclonal B-lymphocytes have recognised an entirely foreign antigen, whereas in fact only the inserted epitope(s) is/are foreign to the host. In this way, antibodies capable of cross-reacting with non-modified self-antigens are induced.

As mentioned above, CTL´s also require specific T cell help, although the mechanism for this is still not clear.

We have based the present invention on our novel theory that the self-proteins containing foreign MHC class II epitopes, following exogenous uptake, can gain access into the MHC class I antigen processing pathway of e.g. macrophages and dendritic cells. In this way a strong CTL response against subdominant epitopes in the self-protein could be induced. Alternatively, genes encoding modified tumour antigens could be administrated as nucleic acid vaccines eventually also leading to MHC class II as well as MHC class I mediated immune responses.

Tumour cells are very poor antigen presenting cells due to insufficient MHC class I expression, lack of co-stimulatory molecules or secretion of immunosuppressive cytokines etc. Using the autovaccine constructs and vaccination protocol mentioned above the modified tumour antigen could be presented by MHC class I as well as by MHC class II molecules on professional antigen presenting cells. Co-presentation of subdominant self-epitopes on MHC class I and immunodominant foreign epitopes on MHC class II molecules would mediate a direct cytokine help from activated MHC class II restricted T-helper cells to MHC class I restricted CTL's (Fig. 2). This will in our opinion lead to a specific break of the T cell autotolerance towards the tumour antigen and this is exactly what is desired in cancer immunotherapy.

Initial CTL experiments where mice have been immunised with dendritic cells pulsed with both a class I and a class II epitope show an enhanced CTL induction when they have been immunised as well as restimulated *in vitro* with both a class I and a class II peptide compared to an immunisation and restimulation with just a class I epitope. This situation is comparable with immunisation with the autovaccine, where a foreign class II epitope is inserted in a self protein. Uptake and processing of these molecules by professional antigen precenting cells such as dendritic cells, leads to presentation of the foreign class II epitope together with some self class I epitopes. It is known that it is possible to elicit autoreactive CTL's, but the presence of a foreign class II helper epitope very likely should enhance this CTL induction.

In conclusion, a vaccine constructed using the technology outlined above will induce a humoral autoantibody response with secondary activation of complement and antibody dependent cellular cytotoxicity (ADCC) activity. It is also expected

that it will induce a cytotoxic T cell response directed against e.g. a tumour specific membrane antigen.

Hence, in the broadest and most general scope, the present invention relates to a method for inducing an immune response against a polypeptide antigen in an animal, including a human being, said polypeptide antigen being weakly immunogenic or non-immunogenic in the animal, the method comprising effecting simultaneous presentation by antigen presenting cells (APCs) from the animal's immune system of an immunogenically effective amount of

1) at least one CTL epitope derived from the polypeptide antigen and/or at least one B-cell epitope derived from the cell-associated polypeptide antigen, and
2) at least one first T helper cell epitope (T_H epitope) which is foreign to the animal.

In a more specific variant of the inventive method, the invention relates to a method for down-regulating a cell-associated polypeptide antigen in an animal, including a human being, said polypeptide antigen being weakly immunogenic or non-immunogenic in the animal, by inducing a specific cytotoxic T-lymphocyte (CTL) response against cells carrying the cell-associated polypeptide antigen on their surface or harbouring the cell-associated polypeptide antigen in their intracellular compartment, the method comprising effecting, in the animal, simultaneous presentation by a suitable antigen presenting cell (APC) of

1) at least one CTL epitope derived from the cell-associated polypeptide antigen, and
2) at least one first T-helper lymphocyte (T_H) epitope which is foreign to the animal.

Also, the novel strategy for preparing an immunogenic agent is part of the invention. This novel strategy encompasses the selection and production of analogues of weak cell-associated antigens, where the preservation of a substantial fraction of known and predicted CTL epitopes is aimed at while at the same time introducing at least one foreign T_H epitope.

Furthermore, the invention relates to certain specific immunogenic constructs based on known tumour-associated antigens as well as to compositions containing these constructs.

Finally, the invention relates to nucleic acid fragments, vectors, transformed cells and other tools useful in molecular biological methods for the production of the analogues of the tumour-associated antigens.

LEGENDS TO THE FIGURE

Fig. 1: The traditional AutoVac concept. A: Tolerodominant self-epitopes presented on MHC class II are ignored due to depletion in the T cell repertoire. B: Inserted foreign immunodominant T cell epitopes presented on MHC class II activate T helper cells. C: B cells specific for native parts of the self-protein presenting foreign immunodominant T cell epitopes on MHC class II are activated by the cytokine help provided by the T helper cell.

Fig. 2: The AutoVac concept for inducing a CTL response. A: Tolerodominant self-epitopes presented on MHC class I are ignored due to depletion in the T cell repertoire. B: Inserted foreign immunodominant T cell epitopes presented on MHC class II activate T helper cells. C: CTL's recognising subdominant self-epitopes presented on MHC class I are activated by the

cytokine help provided by the adjacent activated T helper cell.

Fig. 3: A schematic representation of the Her-2 polypeptide with indications of epitopic regions and N-glycosylation sites. The 4 extracellular domains, the transmembrane (TM) domain and the 2 intracellular domains are represented with indications of sites with varying degrees of homology and sites containing putative/determined CTL epitopes.

Fig. 4: A schematic representation of the human PSM polypeptide with indications of insertion regions for the P2 and P30 epitopes.

Fig. 5: The FGF genes and proteins. A: Exon-intron structure of the human and mouse FGF8 genes. Below is illustrated the eight different splice forms (from Gemel 1996). B: Amino acid sequence of the different FGF8 isoforms. The polypeptide stretches unique to FGF8b, FGF8f, and FGF8e are indicated by bold and italic or underlined typefaces. FGF8a is the shortest variant containing none of these highlighted sequences. The signal peptide is expected to be cleaved C-terminally to Ala22. The two cysteine residues found in mature FGF8(all isoforms) are indicated by thick underlining. The two potential N-glycosylation sites of FGF8b are indicated by wavy underlining. Numbering is according to FGF8b.

Fig. 6: Illustrations of the four different variants of FGF8b designed for autovaccination. Upper panel: Theoretical models of the insertion-points of the epitopes using the FGF2 crystal structure as template. Lower panel: Amino acid sequences of the wild type FGF8b (WT) and the four variants F30N, F2I, F30I, and F2C. The signal peptide is marked with single underlining. The inserted peptides are marked with double underlining. The N-terminal sequence (MetAla) of all variants

is due to generation of a Kozak-sequence (Kozak 1991) for better translation in eucaryotic systems.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the following a number of terms used in the present specification and claims will be defined and explained in detail in order to clarify the metes and bounds of the invention.

A "cell-associated polypeptide antigen" is in the present specification and claims intended to denote a polypeptide which is confined to a cell which is somehow related to a pathological process. Furthermore, the cell presents CTL epitopes of the polypeptide antigen bound to MHC Class I molecules on its surface. Cell-associated polypeptide antigens can therefore be truly intracellular antigens (and thereby unreachable for a humoral immune response) or antigens bound to the surface of the cells. The cell-associated antigen can be the product of the cell's own gene expression, of a intracellular parasite, of a virus, or of another cell. In the latter case the polypeptide antigen is subsequently associated with the cell which is involved in the pathological process.

The terms "T-lymphocyte" and "T-cell" will be used interchangeably for lymphocytes of thymic origin which are responsible for various cell mediated immune responses as well as for effector functions such as helper activity in the humoral immune response. Likewise, the terms "B-lymphocyte" and "B-cell" will be used interchangeably for antibody-producing lymphocytes.

An "antigen presenting cell" (APC) is a cell which presents epitopes to T-cells. Typical antigen-presenting cells are macrophages, dendritic cells and other phagocytizing and pinocytizing cells. It should be noted that B-cells also functions as APCs by presenting T_H epitopes bound to MCH class II molecules to T_H cells but when generally using the term APC in the present specification and claims it is intended to refer to the above-mentioned phagocytizing and pinocytizing cells.

"Helper T-lymphocytes" or "T_H cells" denotes CD4 positive T-cells which provide help to B-cells and cytotoxic T-cells via the recognition of T_H epitopes bound to MHC Class II molecules on antigen presenting cells.

The term "cytotoxic T-lymphocyte" (CTL) will be used for CD8 positive T-cells which require the assistance of T_H cells in order to become activated.

A "specific" immune response is in the present context intended to denote a polyclonal immune response directed predominantly against a molecule or a group of quasi-identical molecules or, alternatively, against cells which present CTL epitopes of the molecule or the group of quasi-identical molecules.

A "weak or non-immunogenic polypeptide antigen" is herein intended to denote polypeptides having the amino acid sequence of the weak cell-associated protein antigens derived from the animal in question (e.g. a human), but also polypeptides having the amino acid sequence identical to analogues of such proteins isolated from other species are embraced by the term. Also forms of the polypeptides having differing glycosylation patterns because of their production in heterologous systems (e.g. yeasts or other non-mammalian eukaryotic expression

systems or even prokaryotic systems) are included within the boundaries of the term. It should, however, be noted that when using the term, it is intended that the polypeptide in question is normally non-immunogenic or only weakly immunogenic in its natural localisation in the animal to be treated.

The term "polypeptide" is in the present context intended to mean both short peptides of from 2 to 10 amino acid residues, oligopeptides of from 11 to 100 amino acid residues, and polypeptides of more than 100 amino acid residues. Furthermore, the term is also intended to include proteins, *i.e.* functional biomolecules comprising at least one polypeptide; when comprising at least two polypeptides, these may form complexes, be covalently linked, or may be non-covalently linked. The polypeptide(s) in a protein can be glycosylated and/or lipidated and/or comprise prosthetic groups.

The term "subsequence" means any consecutive stretch of at least 3 amino acids or, when relevant, of at least 3 nucleotides, derived directly from a naturally occurring amino acid sequence or nucleic acid sequence, respectively.

The term "animal" is in the present context in general intended to denote an animal species (preferably mammalian), such as *Homo sapiens*, *Canis domesticus*, etc. and not just one single animal. However, the term also denotes a population of such an animal species, since it is important that the individuals immunized according to the method of the invention all harbour substantially the same weak, cell-associated polypeptide antigen allowing for immunization of the animals with the same immunogen(s). If, for instance, genetic variants of polypeptides exist in different human populations it may be necessary to use different immunogens in these different

populations in order to be able to break the autotolerance towards the weak, cell-associated polypeptide antigen in each population.

By the term "down-regulation a cell-associated polypeptide antigen" is herein meant reduction in the living organism of the amount and/or activity of the antigen in question. The down-regulation can be obtained by means of several mechanisms: Of these, simple interference with the active site in the antigen by antibody binding is the most simple. However, it is also within the scope of the present invention that the antibody binding results in removal of the polypeptide by scavenger cells (such as macrophages and other phagocytizing cells), and even more important, that cells carrying or harbouring the antigen are killed by CTLs in the animal.

The expression "effecting simultaneous presentation by a suitable APC" is intended to denote that the animal's immune system is subjected to an immunogenic challenge in a controlled manner which results in the simultaneous presentation by APCs of the epitopes in question. As will appear from the disclosure below, such challenge of the immune system can be effected in a number of ways of which the most important are vaccination with polypeptide containing "pharmaccines" (*i.e.* a vaccine which is administered to treat or ameliorate ongoing disease) or nucleic acid "pharmaccine" vaccination. The important result to achieve is that immune competent cells in the animal are confronted with APCs displaying the relevant epitopes in an immunologically effective manner.

The term "immunogenically effective amount" has its usual meaning in the art, *i.e.* an amount of an immunogen which is capable of inducing an immune response which significantly

engages pathogenic agents which share immunological features with the immunogen.

When using the expression that the weak cell-associated polypeptide antigens have been subjected to a "modification" is herein meant a chemical modification of the polypeptide which constitutes the backbone of the polypeptide in question. Such a modification can e.g. be derivatization (e.g. alkylation) of certain amino acid residues in the amino acid sequence, but as will be appreciated from the disclosure below, the preferred modifications comprise changes of the primary structure of the amino acid sequence.

When discussing "tolerance" and "autotolerance" is understood that since the polypeptides which are the targets of the present inventive method are self-proteins in the population to be vaccinated or proteins which do not result in induction of an effective immune response, normal individuals in the population do not mount an immune response against the polypeptide. It cannot be excluded, though, that occasional individuals in an animal population might be able to produce antibodies against the native polypeptide antigen, e.g. as part of a autoimmune disorder. At any rate, an animal will normally only be autotolerant towards its own polypeptide antigen, but it cannot be excluded that analogues derived from other animal species or from a population having a different phenotype would also be tolerated by said animal.

A "foreign T-cell epitope" is a peptide which is able to bind to an MHC molecule and stimulates T-cells in an animal species. Preferred foreign epitopes are "promiscuous" epitopes, *i.e.* epitopes which binds to a substantial fraction of MHC class II molecules in an animal species or population. Only a very limited number of such promiscuous T-cell epitopes are known, and they will be discussed in detail below. It

should be noted that in order for the immunogens which are used according to the present invention to be effective in as large a fraction of an animal population as possible, it may be necessary to 1) insert several foreign T-cell epitopes in the same analogue or 2) prepare several analogues wherein each analogue has a different promiscuous epitope inserted. It should be noted that the concept of foreign T-cell epitopes also encompasses use of cryptic T-cell epitopes, *i.e.* epitopes which are derived from a self-protein and which only exerts immunogenic behaviour when existing in isolated form without being part of the self-protein in question.

A "CTL" epitope is a peptide which is able to bind to an MHC class I molecule.

A "functional part" of a (bio)molecule is in the present context intended to mean the part of the molecule which is responsible for at least one of the biochemical or physiological effects exerted by the molecule. It is well-known in the art that many enzymes and other effector molecules have an active site which is responsible for the effects exerted by the molecule in question. Other parts of the molecule may serve a stabilizing or solubility enhancing purpose and can therefore be left out if these purposes are not of relevance in the context of a certain embodiment of the present invention. For instance it is possible to use certain cytokines as a modifying moiety in the analogue (cf. the detailed discussion below), and in such a case, the issue of stability may be irrelevant since the coupling to the analogue provides the stability necessary.

Preferred embodiments

In order to induce a CTL response against a cell which presents epitopes derived from the polypeptide antigen on its

surface, it is normally necessary that at least one CTL epitope, when presented, is associated with an MHC Class I molecule on the surface of the APC. Furthermore it is preferred that the at least one first foreign T_H epitope, when presented, is associated with an MHC Class II molecule on the surface of the APC.

Preferred APCs presenting the epitopes are dendritic cells and macrophages, but any pino- or phagocytizing APC which is capable of simultaneously presenting 1) CTL epitopes bound to MHC class I molecules and 2) T_H epitopes bound to MHC class II molecules, is a preferred APC according to the invention.

According to the invention, the cell-associated antigen is preferably selected from a tumour-associated antigens and other self-proteins which are related to pathological processes but also viral antigens and antigens derived from an intracellular parasite or bacterium will. It is well-known in the art that such pathogen-associated antigens are often relatively poor immunogens (e.g. antigens from mycobacteria such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*, but also from protozoans such as *Plasmodium spp.*). It is believed that the method of the invention, apart from rendering possible the production of antibody and CTL responses against true self-protein antigens, is capable of enhancing the often insufficient immune response mounted by the organism against such intracellular antigens.

Normally, it will be advantageous to confront the immune system with a large fraction of the amino acid sequence of the polypeptide antigen which is the vaccine target. Hence, in a preferred embodiment, presentation by the APC of the CTL epitope and the first foreign T_H epitope is effected by presenting the animal's immune system with at least one first analogue of the cell-associated polypeptide antigen, said

first analogue comprising a variation of the amino acid sequence of the cell-associated polypeptide antigen, said variation containing at least the CTL epitope and the first foreign T_H epitope. This is in contrast to e.g. a DNA vaccination strategy where the CTL and T_H epitopes are expressed by the same cell but as parts of separate polypeptides; such a DNA vaccination strategy is also an embodiment of the invention, but it is believed that having the two epitopes as part of the same polypeptide will normally enhance the immune response and, at any rate, only one expression product will be necessary.

In order to maximize the chances of mounting an effective immune response, it is preferred that the above-mentioned first analogue contains a substantial fraction of known and predicted CTL epitopes of the cell-associated polypeptide antigen, *i.e.* a fraction of the known and predicted CTL epitopes which binds a sufficient fractions of MHC Class I molecules in a population. For instance, it is preferred that the substantial fraction of known and predicted CTL epitopes in the amino acid sequence of the analogue are recognized by at least 50% of the MHC-I haplotypes recognizing all known and predicted CTL epitopes in the cell-associated polypeptide antigen, but higher percentages are preferred, such as at least 60, at least 70, at least 80, and at least 90%. Especially preferred is the use of analogues which preserves substantially all known CTL epitopes of the cell-associated polypeptide antigen are present in the analogue, *i.e.* close to 100% of the known CTL epitopes. Accordingly, it is also especially preferred that substantially all predicted CTL epitopes of the cell-associated polypeptide antigen are present in the at least first analogue.

Methods for predicting the presence of CTL epitopes are well-known in the art, cf. the below discussion of specific

algorithms used for the identification of putative MHC I binding peptide fragments.

As will be apparent from the present specification and claims it is expected that the inventive method described herein will render possible the effective induction of CTL responses against cell-associated polypeptide antigens.

In cases where the cell-associated polypeptide antigen is truly intracellular, the induction of a CTL response against cells harbouring the antigen is the only way to achieve its down-regulation by specific immunological means. However, in the case of membrane-associated antigens, it is advantageous to induce a antibody response against the weak, cell-associated polypeptide antigen.

This can be achieved in a number of ways known to the person skilled in the art. For instance, the at least one first analogue may, apart from the at least one CTL epitope and the at least one foreign T_H epitope, comprise a part consisting of a modification of the structure of the cell-associated polypeptide antigen, said modification having as a result that immunization of the animal with the first analogue induces production of antibodies in the animal against the cell-associated polypeptide antigen. Alternatively, the method of the invention can involve effecting presentation to the animal's immune system of an immunogenically effective amount of at least one *second* analogue of the cell-associated polypeptide antigen which contains such a modification. A convenient way to achieve that the modification has the desired antibody-inducing effect is to include at least one second foreign T_H epitope in the second analogue, *i.e.* a strategy like the one used for the first analogue.

In the cases where it is desired to also mount an effective humoral immune response, it is advantageous that the first and/or second analogue(s) comprise(s) a substantial fraction of the cell-associated polypeptide antigen's B-cell epitopes.

The above-discussed variations and modifications of the weak, cell-associated polypeptide antigen can take different forms. It is preferred that the variation and/or modification involves amino acid substitution and/or deletion and/or insertion and/or addition. These fundamental operations relating to the manipulation of an amino acid sequence are intended to cover both single-amino acid changes as well as operations involving stretches of amino acids (*i.a.* shuffling of amino acid stretches within the polypeptide antigen; this is especially interesting when the antigen is a true intracellular antigen, since only considerations concerning preservation of CTL epitopes are relevant). It will be understood, that the introduction of e.g. one single amino acid insertion or deletion may give rise to the emergence of a foreign T_H epitope in the sequence of the analogue, *i.e.* the emergence of an MHC Class II molecule binding sequence. However, in most situations it is preferable (and even necessary) to introduce a known foreign T_H epitope, and such an operation will require acid substitution and/or insertion (or sometimes addition in the form of either conjugation to a carrier protein or provision of a fusion polypeptide by means of molecular biology methods. It is preferred that the number of amino acid insertions, deletions, substitutions or additions is at least 2, such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, and 25 insertions, substitutions, additions or deletions. It is furthermore preferred that the number of amino acid substitutions is not in excess of 150, such as at most 100, at most 90, at most 80, and at most 70. It is especially preferred that the number of substitutions, insertions, deletions, or additions does not

exceed 60, and in particular the number should not exceed 50 or even 40. Most preferred is a number of not more than 30.

Preferred embodiments of the invention includes modification by introducing at least one foreign immunodominant T_H epitope. It will be understood that the question of immune dominance of a T-cell epitope depends on the animal species in question. As used herein, the term "immunodominance" simply refers to epitopes which in the vaccinated individual gives rise to a significant immune response, but it is a well-known fact that a T-cell epitope which is immunodominant in one individual is not necessarily immunodominant in another individual of the same species, even though it may be capable of binding MHC-II molecules in the latter individual.

Another important point is the issue of MHC restriction of T-cell epitopes. In general, naturally occurring T-cell epitopes are MHC restricted, *i.e.* a certain peptides constituting a T-cell epitope will only bind effectively to a subset of MHC Class II molecules. This in turn has the effect that in most cases the use of one specific T-cell epitope will result in a vaccine component which is only effective in a fraction of the population, and depending on the size of that fraction, it can be necessary to include more T-cell epitopes in the same molecule, or alternatively prepare a multi-component vaccine wherein the components are OPGL variants which are distinguished from each other by the nature of the T-cell epitope introduced.

If the MHC restriction of the T-cells used is completely unknown (for instance in a situation where the vaccinated animal has a poorly defined MHC composition), the fraction of the population covered by a specific vaccine composition can be determined by means of the following formula

$$f_{population} = 1 - \prod_{i=1}^{n} (1 - p_i) \qquad \text{(II)}$$

-where p_i is the frequency in the population of responders to the i^{th} foreign T-cell epitope present in the vaccine composition, and n is the total number of foreign T-cell epitopes in the vaccine composition. Thus, a vaccine composition containing 3 foreign T-cell epitopes having response frequencies in the population of 0.8, 0.7, and 0.6, respectively, would give

$$1 - 0.2 \times 0.3 \times 0.4 = 0.976$$

-*i.e.* 97.6 percent of the population will statistically mount an MHC-II mediated response to the vaccine.

The above formula does not apply in situations where a more or less precise MHC restriction pattern of the peptides used is known. If, for instance a certain peptide only binds the human MHC-II molecules encoded by HLA-DR alleles DR1, DR3, DR5, and DR7, then the use of this peptide together with another peptide which binds the remaining MHC-II molecules encoded by HLA-DR alleles will accomplish 100% coverage in the population in question. Likewise, if the second peptide only binds DR3 and DR5, the addition of this peptide will not increase the coverage at all. If one bases the calculation of population response purely on MHC restriction of T-cell epitopes in the vaccine, the fraction of the population covered by a specific vaccine composition can be determined by means of the following formula:

$$f_{population} = 1 - \prod_{j=1}^{3} (1 - \varphi_j)^2 \qquad \text{(III)}$$

-wherein φ_j is the sum of frequencies in the population of allelic haplotypes encoding MHC molecules which bind any one of the T-cell epitopes in the vaccine and which belong to the j^{th} of the 3 known HLA loci (DP, DR and DQ); in practice, it is first determined which MHC molecules will recognize each T-cell epitope in the vaccine and thereafter these are listed by type (DP, DR and DQ) - then, the individual frequencies of the different listed allelic haplotypes are summed for each type, thereby yielding φ_1, φ_2, and φ_3.

It may occur that the value p_i in formula II exceeds the corresponding theoretical value π_i:

$$\pi_i = 1 - \prod_{j=1}^{3} (1 - \nu_j)^2 \qquad \text{(IV)}$$

-wherein υ_j is the sum of frequencies in the population of allelic haplotype encoding MHC molecules which bind the i^{th} T-cell epitope in the vaccine and which belong to the j^{th} of the 3 known HLA loci (DP, DR and DQ). This means that in $1-\pi_i$ of the population is a frequency of responders of $f_{residual\_i} = (p_i - \pi_i)/(1-\pi_i)$. Therefore, formula III can be adjusted so as to yield formula V:

$$f_{population} = 1 - \prod_{j=1}^{3} (1 - \varphi_j)^2 + \left(1 - \prod_{i=1}^{n} (1 - f_{residual\_i})\right) \qquad \text{(V)}$$

-where the term $1-f_{residual-i}$ is set to zero if negative. It should be noted that formula V requires that all epitopes have been haplotype mapped against identical sets of haplotypes.

Therefore, when selecting T-cell epitopes to be introduced in the OPGL analogue, it is important to include all knowledge of the epitopes which is available: 1) The frequency of responders in the population to each epitope, 2) MHC

restriction data, and 3) frequency in the population of the relevant haplotypes.

There exist a number of naturally occurring "promiscuous" T-cell epitopes which are active in a large proportion of individuals of an animal species or an animal population and these are preferably introduced in the vaccine thereby reducing the need for a very large number of different OPGL analogues in the same vaccine.

The promiscuous epitope can according to the invention be a naturally occurring human T-cell epitope such as epitopes from tetanus toxoid (e.g. the P2 and P30 epitopes), diphtheria toxoid, Influenza virus hemagluttinin (HA), and P. falciparum CS antigen.

Over the years a number of other promiscuous T-cell epitopes have been identified. Especially peptides capable of binding a large proportion of HLA-DR molecules encoded by the different HLA-DR alleles have been identified and these are all possible T-cell epitopes to be introduced in the OPGL analogues used according to the present invention. Cf. also the epitopes discussed in the following references which are hereby all incorporated by reference herein: Southwood S et. al, 1998, J. Immunol. 160: 3363-3373; Sinigaglia F et al., 1988, Nature 336: 778-780; Chicz RM et al., 1993, J. Exp. Med 178: 27-47; Hammer J et al., 1993, Cell 74: 197-203; and Falk K et al., 1994, Immunogenetics 39: 230-242. The latter reference also deals with HLA-DQ and -DP ligands. All epitopes listed in these 5 references are relevant as candidate natural epitopes to be used in the present invention, as are epitopes which share common motifs with these.

Alternatively, the epitope can be any artificial T-cell epitope which is capable of binding a large proportion of

haplotypes. In this context the pan DR epitope peptides ("PADRE") described in WO 95/07707 and in the corresponding paper Alexander J et al., 1994, Immunity 1: 751-761 (both disclosures are incorporated by reference herein) are interesting candidates for epitopes to be used according to the present invention. It should be noted that the most effective PADRE peptides disclosed in these papers carry D-amino acids in the C- and N-termini in order to improve stability when administered. However, the present invention primarily aims at incorporating the relevant epitopes as part of the modified OPGL which should then subsequently be broken down enzymatically inside the lysosomal compartment of APCs to allow subsequent presentation in the context of an MHC-II molecule and therefore it is not expedient to incorporate D-amino acids in the epitopes used in the present invention.

One especially preferred PADRE peptide is the one having the amino acid sequence AKFVAAWTLKAAA or an immunologically effective subsequence thereof. This, and other epitopes having the same lack of MHC restriction are preferred T-cell epitopes which should be present in the OPGL analogues used in the inventive method. Such super-promiscuous epitopes will allow for the most simple embodiments of the invention wherein only one single analogue is presented to the vaccinated animal's immune system.

The nature of the above-discussed variation/modification preferably comprises that

- at least one first moiety is included in the first and/or second analogue(s), said first moiety effecting targeting of the analogue to an antigen presenting cell (APC), and/or
- at least one second moiety is included in the first and/or second analogue(s), said second moiety stimulating the immune system, and/or

- at least one third moiety is included in the first and/or second analogue(s), said third moiety optimizing presentation of the analogue to the immune system.

The functional and structural features relating these first, second and third moieties will be discussed in the following:

They can be present in the form of side groups attached covalently or non-covalently to suitable chemical groups in the amino acid sequence of the cell-associated polypeptide antigen or a subsequence thereof. This is to mean that stretches of amino acid residues derived from the polypeptide antigen are derivatized without altering the primary amino acid sequence, or at least without introducing changes in the peptide bonds between the individual amino acids in the chain.

The moieties can also be in the form of fusion partners to the amino acid sequence derived from the cell-associated polypeptide antigen. In this connection it should be mentioned that both possibilities include the option of conjugating the amino acid sequence to a carrier, cf. the discussion of these below. In other words, in the present context the term "fusion protein is not merely restricted to a fusion construct prepared by means of expression of a DNA fragment encoding the construct but also to a conjugate between two proteins which are joined by means of a peptide bond in a subsequent chemical reaction.

As mentioned above, the analogue can also include the introduction of a first moiety which targets the analogue to an APC or a B-lymphocyte. For instance, the first moiety can be a specific binding partner for a B-lymphocyte specific surface antigen or for an APC specific surface antigen. Many such specific surface antigens are known in the art. For instance, the moiety can be a carbohydrate for which there is

a receptor on the B-lymphocyte or the APC (e.g. mannan or mannose). Alternatively, the second moiety can be a hapten. Also an antibody fragment which specifically recognizes a surface molecule on APCs or lymphocytes can be used as a first moiety (the surface molecule can e.g. be an FCγ receptor of macrophages and monocytes, such as FCγRI or, alternatively any other specific surface marker such as CD40 or CTLA-4). It should be noted that all these exemplary targeting molecules can be used as part of an adjuvant, cf. below. CD40 ligand, antibodies against CD40, or variants thereof which bind CD40 will target the analogue to dendritic cells. At the same time, recent results have shown that the interaction with the CD40 molecule renders the T_H cells unessential for obtaining a CTL response. Hence, it is contemplated that the general use of CD40 binding molecules as the first moiety (or as adjuvants, cf. below) will enhance the CTL response considerably; in fact, the use of such CD40 binding molecules as adjuvants and "first moieties" in the meaning of the present invention is believed to be inventive in its own right.

As an alternative or supplement to targeting the analogue to a certain cell type in order to achieve an enhanced immune response, it is possible to increase the level of responsiveness of the immune system by including the above-mentioned second moiety which stimulates the immune system. Typical examples of such second moieties are cytokines, and heat-shock proteins, as well as effective parts thereof.

Suitable cytokines to be used according to the invention are those which will normally also function as adjuvants in a vaccine composition, e.g. interferon γ (IFN-γ), Flt3 ligand (Flt3L), interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin 13 (IL-13), interleukin 15 (IL-15), and granulocyte-macrophage colony stimulating factor (GM-CSF);

alternatively, the functional part of the cytokine molecule may suffice as the second moiety. With respect to the use of such cytokines as adjuvant substances, cf. the discussion below.

Alternatively, the second moiety can be a toxin, such as listeriolycin (LLO), lipid A and heat-labile enterotoxin. Also, a number of mycobacterial derivatives such as MDP (muramyl dipeptide), CFA (complete Freund's adjuvant) and the trehalose diesters TDM and TDE are interesting possibilities.

According to the invention, suitable heat shock proteins used as the second moiety can be HSP70, HSP90, and HSC70.

Also the possibility of introducing a third moiety which enhances the presentation of the analogue to the immune system is an important embodiment of the invention. The art has shown several examples of this principle. For instance, it is known that the palmitoyl lipidation anchor in the Borrelia burgdorferi protein OspA can be utilised so as to provide self-adjuvating polypeptides (cf. e.g. WO 96/40718). It seems that the lipidated proteins form up micelle-like structures with a core consisting of the lipidation anchor parts of the polypeptides and the remaining parts of the molecule protruding therefrom, resulting in multiple presentations of the antigenic determinants. Hence, the use of this and related approaches using different lipidation anchors (e.g. a myristyl anchor) are preferred embodiments of the invention, especially since the provision of such a lipidation anchor in a recombinantly produced protein is fairly straightforward and merely requires use of e.g. a naturally occurring signal sequence as a fusion partner for the analogue. Another possibility is use of the C3d fragment of complement factor C3 or C3 itself (cf. Dempsey et al., 1996, Science 271, 348-350 and Lou & Kohler, 1998, Nature Biotechnology 16, 458-462).

It is important to note that when attempting to use the method of the invention against e.g. membrane bound polypeptide antigens which are exposed to the extracellular compartment, it is most preferred that the first and/or second analogue(s) has/have substantially the overall tertiary structure of the cell-associated polypeptide antigen. In the present specification and claims this is intended to mean that the overall tertiary structure of the *part* of the polypeptide antigen which is extracellularly exposed is preserved, since, as mentioned above, the tertiary structure of the obligate intracellular polypeptides do not engage the humeral immune system. In fact, as part of the vaccination strategy it is often desired to avoid exposure to the extracellular compartment of putative B-cell epitopes derived from intracellular part of the polypeptide antigens; in this way, potentially adverse effects caused by cross-reactivity with other antigens can be minimized.

For the purposes of the present invention, it is however sufficient if the variation/modification (be it an insertion, addition, deletion or substitution) gives rise to a foreign T-cell epitope and at the same time preserves a substantial number of the CTL epitopes in the polypeptide antigen (and sometimes also a substantial number of B-cell epitopes). The following formula describes the constructs generally covered by the invention:

$$(MOD_1)_{s1}(PAG_{e1})_{n1}(MOD_2)_{s2}(PAG_{e2})_{n2}\ldots(MOD_x)_{sx}(PAG_{ex})_{nx} \qquad (I)$$

-where PAG_{e1}-PAG_{ex} are x CTL and/or B-Cell epitope containing subsequences of the relevant polypeptide antigen which independently are identical or non-identical and which may contain or not contain foreign side groups, x is an integer ≥ 3, n1-nx are x integers ≥ 0 (at least one is ≥ 1), MOD_1-MOD_x are x modifications introduced between the preserved epitopes,

and s1-sx are x integers ≥ 0 (at least one is ≥ 1 if no side groups are introduced in the sequences). Thus, given the general functional restraints on the immunogenicity of the constructs, the invention allows for all kinds of permutations of the original antigen sequence, and all kinds of modifications therein. Thus, included in the invention are analogues obtained by omission of parts of the polypeptide antigen sequence which e.g. exhibit adverse effects in vivo or omission of parts which are normally intracellular and thus could give rise to undesired immunological reactions, cf. the detailed discussion below.

It is furthermore preferred that the variation and/or modification includes duplication, when applicable, of the at least one B-cell epitope, or of at least one CTL epitope of the cell-associated polypeptide antigen. This strategy will give the result that multiple copies of preferred epitopic regions are presented to the immune system and thus maximizing the probability of an effective immune response. Hence, this embodiment of the invention utilises multiple presentations of epitopes derived from the polypeptide antigen (*i.e.* formula I wherein at least one B-cell epitope is present in two positions).

This effect can be achieved in various ways, e.g. by simply preparing fusion polypeptides comprising the structure $(PAG)_m$, where m is an integer ≥ 2 and then introduce the modifications discussed herein in at least one of the polypeptide antigen sequences. It is preferred that the modifications introduced includes at least one duplication of a B-lymphocyte epitope and/or the introduction of a hapten.

An alternative embodiment of the invention which also results in the preferred presentation of multiple (e.g. at least 2) copies of the important epitopic regions of OPGL to the immune

system is the covalent coupling of OPGL, subsequence or variants thereof to certain molecules. For instance, polymers can be used, e.g. carbohydrates such as dextran, cf. e.g. Lees A et al., 1994, Vaccine 12: 1160-1166; Lees A et al., 1990, J Immunol. 145: 3594-3600, but also mannose and mannan are useful alternatives. Integral membrane proteins from e.g. *E. coli* and other bacteria are also useful conjugation partners. The traditional carrier molecules such as keyhole limpet haemocyanin (KLH), tetanus toxoid, diphtheria toxoid, and bovine serum albumin (BSA) are also preferred and useful conjugation partners.

Maintenance of the sometimes advantageous substantial fraction of B-cell epitopes or even the overall tertiary structure of a protein which is subjected to modification as described herein can be achieved in several ways. One is simply to prepare a polyclonal antiserum directed against the polypeptide antigen (e.g. an antiserum prepared in a rabbit) and thereafter use this antiserum as a test reagent (e.g. in a competitive ELISA) against the modified proteins which are produced. Modified versions (analogues) which react to the same extent with the antiserum as does the polypeptide antigen must be regarded as having the same overall tertiary structure as the polypeptide antigen whereas analogues exhibiting a limited (but still significant and specific) reactivity with such an antiserum are regarded as having maintained a substantial fraction of the original B-cell epitopes.

Alternatively, a selection of monoclonal antibodies reactive with distinct epitopes on the polypeptide antigen can be prepared and used as a test panel. This approach has the advantage of allowing 1) an epitope mapping of the polypeptide antigen in question and 2) a mapping of the epitopes which are maintained in the analogues prepared.

Of course, a third approach would be to resolve the 3-dimensional structure of the polypeptide antigen or of a biologically active truncate thereof (cf. above) and compare this to the resolved three-dimensional structure of the analogues prepared. Three-dimensional structure can be resolved by the aid of X-ray diffraction studies, circular dichroism studies, NMR-spectroscopy etc. Of these, circular dichroism has the advantage of merely requiring the polypeptide in pure form (whereas X-ray diffraction requires the provision of crystallized polypeptide and NMR requires the provision of isotopic variants of the polypeptide) in order to provide useful information about the tertiary structure of a given molecule. However, ultimately X-ray diffraction and/or NMR are necessary to obtain conclusive data since circular dichroism only provides indirect evidence of correct 3-dimensional structure via information of secondary structure elements.

In essence there are three feasible ways of obtaining the presentation of the relevant epitopes to the immune system: Traditional sub-unit vaccination with polypeptide antigens, administration of a genetically modified live vaccine, and nucleic acid vaccination. These three possibilities will be discussed separately in the following:

Polypeptide vaccination

This entails administration to the animal in question of an immunogenically effective amount of the at least one first analogue, and, when relevant, administration of an immunologically effective amount of the at least one second analogue. Preferably, the at least one first and/or second analogue(s) is/are formulated together with a pharmaceutically and immunologically acceptable carrier and/or vehicle and/or diluent and/or excipient and, optionally an adjuvant.

When effecting presentation of the analogue to an animal's immune system by means of administration thereof to the animal, the formulation of the polypeptide follows the principles generally acknowledged in the art.

Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by US Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as injectables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines; cf. the detailed discussion of adjuvants below.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously, intradermally, subdermally or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral, buccal, sublinqual, intraperitoneal, intravaginal, anal and intracranial formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of

mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%. For oral formulations, cholera toxin is an interesting formulation partner (and also a possible conjugation partner).

The polypeptides may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to mount an immune response, and the degree of protection desired. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1 µg to 2000 µg (even though higher amounts in the 1-10 mg range are contemplated), such as in the range from about 0.5 µg to 1000 µg, preferably in the range from 1 µg to 500 µg and especially in the range from about 10 µg to 100 µg. Suitable regimens for initial administration and booster shots are also variable but are typified by an initial

administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the age of the person to be vaccinated and the formulation of the antigen.

Some of the polypeptides of the vaccine are sufficiently immunogenic in a vaccine, but for some of the others the immune response will be enhanced if the vaccine further comprises an adjuvant substance. It is especially preferred to use an adjuvant which can be demonstrated to facilitate breaking of the autotolerance to autoantigens.

Various methods of achieving adjuvant effect for the vaccine are known. General principles and methods are detailed in "The Theory and Practical Application of Adjuvants", 1995, Duncan E.S. Stewart-Tull (ed.), John Wiley & Sons Ltd, ISBN 0-471-95170-6, and also in "Vaccines: New Generationn Immunological Adjuvants", 1995, Gregoriadis G et al. (eds.), Plenum Press, New York, ISBN 0-306-45283-9, both of which are hereby incorporated by reference herein.

Preferred adjuvants facilitate uptake by APCs, such as dendritic cells, of the at least first and/or second analogues. Non-limiting examples are selected from the group consisting of an immune targeting adjuvant; an immune modulating adjuvant such as a toxin, a cytokine, and a mycobacterial derivative; an oil formulation; a polymer; a micelle forming adjuvant; a saponin; an immunostimulating

complex matrix (ISCOM matrix); a particle; DDA; aluminium adjuvants; DNA adjuvants; γ-inulin; and an encapsulating adjuvant. In general it should be noted that the disclosures above which relate to compounds and agents useful as first, second and third moieties in the analogues also refer *mutatis mutandis* to their use in the adjuvant of a vaccine of the invention.

The application of adjuvants include use of agents such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in buffered saline, admixture with synthetic polymers of sugars (e.g. Carbopol®) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70° to 101°C for 30 second to 2 minute periods respectively and also aggregation by means of cross-linking agents are possible. Aggregation by reactivation with pepsin treated antibodies (Fab fragments) to albumin, mixture with bacterial cells such as C. parvum or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed. Admixture with oils such as squalene and IFA is also preferred.

According to the invention DDA (dimethyldioctadecylammonium bromide) is an interesting candidate for an adjuvant as is DNA and γ-inulin, but also Freund's complete and incomplete adjuvants as well as quillaja saponins such as QuilA and QS21 are interesting as is RIBI. Further possibilities are monophosphoryl lipid A (MPL), the above mentioned C3 and C3d, and muramyl dipeptide (MDP).

Liposome formulations are also known to confer adjuvant effects, and therefore liposome adjuvants are preferred according to the invention.

Also immunostimulating complex matrix type (ISCOM® matrix) adjuvants are preferred choices according to the invention, especially since it has been shown that this type of adjuvants are capable of up-regulating MHC Class II expression by APCs. An ISCOM® matrix consists of (optionally fractionated) saponins (triterpenoids) from Quillaja saponaria, cholesterol, and phospholipid. When admixed with the immunogenic protein, the resulting particulate formulation is what is known as an ISCOM particle where the saponin constitutes 60-70% w/w, the cholesterol and phospholipid 10-15% w/w, and the protein 10-15% w/w. Details relating to composition and use of immunostimulating complexes can e.g. be found in the above-mentioned text-books dealing with adjuvants, but also Morein B et al., 1995, Clin. Immunother. 3: 461-475 as well as Barr IG and Mitchell GF, 1996, Immunol. and Cell Biol. 74: 8-25 (both incorporated by reference herein) provide useful instructions for the preparation of complete immunostimulating complexes. Another highly interesting (and thus, preferred) possibility of achieving adjuvant effect is to employ the technique described in Gosselin et al., 1992 (which is hereby incorporated by reference herein). In brief, the presentation of a relevant antigen such as an antigen of the present invention can be enhanced by conjugating the antigen to antibodies (or antigen binding antibody fragments) against the Fcγ receptors on monocytes/macrophages. Especially conjugates between antigen and anti-FcγRI have been demonstrated to enhance immunogenicity for the purposes of vaccination.

Other possibilities involve the use of the targeting and immune modulating substances (i.a. cytokines) mentioned above as candidates for the first and second moieties in the

modified analogues. In this connection, also synthetic inducers of cytokines like poly I:C are possibilities.

Suitable mycobacterial derivatives are selected from the group consisting of muramyl dipeptide, complete Freund's adjuvant, RIBI, and a diester of trehalose such as TDM and TDE.

Suitable immune targeting adjuvants are selected from the group consisting of CD40 ligand and CD40 antibodies or specifically binding fragments thereof (cf. the discussion above), mannose, a Fab fragment, and CTLA-4.

Suitable polymer adjuvants are selected from the group consisting of a carbohydrate such as dextran, PEG, starch, mannan, and mannose; a plastic polymer such as; and latex such as latex beads.

Recent findings that the co-administration of H2 agonists enhances the in-tumour survival of Natural Killer Cells and CTLs. Hence, it is also contemplated to include H2 agonists as adjuvants in the methods of the invention.

It is expected that the vaccine should be administered at least once a year, such as at least 1, 2, 3, 4, 5, 6, and 12 times a year. More specifically, 1-12 times per year is expected, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 times a year to an individual in need thereof. It has previously been shown that the memory immunity induced by the use of the preferred autovaccines according to the invention is not permanent, and therefor the immune system needs to be periodically challenged with the analogues.

Due to genetic variation, different individuals may react with immune responses of varying strength to the same polypeptide. Therefore, the vaccine according to the invention may comprise

several different polypeptides in order to increase the immune response, cf. also the discussion above concerning the choice of foreign T-cell epitope introductions. The vaccine may comprise two or more polypeptides, where all of the polypeptides are as defined above.

The vaccine may consequently comprise 3-20 different modified or unmodified polypeptides, such as 3-10 different polypeptides. However, normally the number of peptides will be sought kept to a minimum such as 1 or 2 peptides.

Live vaccines

The second alternative for effecting presentation to the immune system is the use of live vaccine technology. In live vaccination, presentation to the immune system is effected by administering, to the animal, a non-pathogenic microorganism which has been transformed with a nucleic acid fragment encoding the necessary epitopic regions or a complete 1st and/or 2nd analogue. Alternatively, the microorganism is transformed with a vector incorporating such a nucleic acid fragment. The non-pathogenic microorganism can be any suitable attenuated bacterial strain (attenuated by means of passaging or by means of removal of pathogenic expression products by recombinant DNA technology), e.g. *Mycobacterium bovis* BCG., non-pathogenic *Streptococcus* spp., *E. coli*, *Salmonella* spp., *Vibrio cholerae*, *Shigella*, etc. Reviews dealing with preparation of state-of-the-art live vaccines can e.g. be found in Saliou P, 1995, Rev. Prat. 45: 1492-1496 and Walker PD, 1992, Vaccine 10: 977-990, both incorporated by reference herein. For details about the nucleic acid fragments and vectors used in such live vaccines, cf. the discussion below.

As for the polypeptide vaccine, the T_H epitope and/or the first and/or second and/or third moieties can, if present, be in the form of fusion partners to the amino acid sequence derived from the cell-associated polypeptide antigen.

As an alternative to bacterial live vaccines, the nucleic acid fragment of the invention discussed below can be incorporated in a non-virulent viral vaccine vector such as a vaccinia strain.

Normally, the non-pathogenic microorganism or virus is administered only once to the animal, but in certain cases it may be necessary to administer the microorganism more than once in a lifetime.

Also, the microorganism can be transformed with nucleic acid(s) containing regions encoding the 1st, 2nd and/or 3rd moieties, e.g. in the form of the immunomodulating substances described above such as the cytokines discussed as useful adjuvants. A preferred version of this embodiment encompasses having the coding region for the analogue and the coding region for the immunomodulator in different reading frames or at least under the control of different promoters. Thereby it is avoided that the analogue or epitopes are produced as fusion partners to the immunomodulator. Alternatively, two distinct nucleotide fragments can be used as transforming agents.

Nucleic acid vaccination

As an alternative to classic administration of a peptide-based vaccine, the technology of nucleic acid vaccination (also known as "nucleic acid immunisation", "genetic immunisation", "gene immunisation" and "DNA vaccination) offers a number of attractive features.

First, in contrast to the traditional vaccine approach, nucleic acid vaccination does not require resource consuming large-scale production of the immunogenic agent (e.g. in the form of industrial scale fermentation of microorganisms producing the analogues necessary in polypeptide vaccination). Furthermore, there is no need to device purification and refolding schemes for the immunogen. And finally, since nucleic acid vaccination relies on the biochemical apparatus of the vaccinated individual in order to produce the expression product of the nucleic acid introduced, the optimum posttranslational processing of the expression product is expected to occur; this is especially important in the case of autovaccination, since, as mentioned above, a significant fraction of the original B-cell epitopes should be preserved in the analogues derived from extracellularly exposed polypeptide sequences, and since B-cell epitopes in principle can be constituted by parts of any (bio)molecule (e.g. carbohydrate, lipid, protein etc.). Therefore, native glycosylation and lipidation patterns of the immunogen may very well be of importance for the overall immunogenicity and this is best ensured by having the host producing the immunogen.

Hence, an important embodiment of the method of the invention involves that presentation is effected by *in vivo* introducing, into the APC, at least one nucleic acid fragment which encodes and expresses the at least one CTL epitope and/or the at least one B-cell epitope, and the at least one first foreign T_H epitope (an alternative encompasses administration of at least 2 distinct nucleic acid fragments, where one encodes the at least one CTL epitope and the other encodes the at least one foreign T_H epitope). Preferably, this is done by using a nucleic acid fragment which encodes and expresses the above-discussed first analogue. If the first analogue is equipped with the above-detailed T_H epitopes and/or first and/or second

and/or third moieties, these are then present in the form of fusion partners to the amino acid sequence derived from the cell-associated polypeptide antigen, the fusion construct being encoded by the nucleic acid fragment.

As for the traditional vaccination approach, the nucleic acid vaccination can be combined with *in vivo* introduction, into the APC, of at least one nucleic acid fragment encoding and expressing the second analogue. The considerations pertaining to 1st, 2nd and 3rd moieties and T_H epitopes apply also here.

In this embodiment, the introduced nucleic acid is preferably DNA which can be in the form of naked DNA, DNA formulated with charged or uncharged lipids, DNA formulated in liposomes, DNA included in a viral vector, DNA formulated with a transfection-facilitating protein or polypeptide, DNA formulated with a targeting protein or polypeptide, DNA formulated with Calcium precipitating agents, DNA coupled to an inert carrier molecule, and DNA formulated with an adjuvant. In this context it is noted that practically all considerations pertaining to the use of adjuvants in traditional vaccine formulation apply for the formulation of DNA vaccines. Hence, all disclosures herein which relate to use of adjuvants in the context of polypeptide based vaccines apply *mutatis mutandis* to their use in nucleic acid vaccination technology. The same holds true for other considerations relating to formulation and mode and route of administration and, hence, also these considerations discussed above in connection with a traditional vaccine apply *mutatis mutandis* to their use in nucleic acid vaccination technology.

Furthermore, the nucleic acid(s) used as an immunization agent can contain regions encoding the 1st, 2nd and/or 3rd moieties, e.g. in the form of the immunomodulating substances described above such as the cytokines discussed as useful adjuvants. A

preferred version of this embodiment encompasses having the coding region for the analogue and the coding region for the immunomodulator in different reading frames or at least under the control of different promoters. Thereby it is avoided that the analogue or epitope is produced as a fusion partner to the immunomodulator. Alternatively, two distinct nucleotide fragments can be used, but this is less preferred because of the advantage of ensured co-expression when having both coding regions included in the same molecule.

Under normal circumstances, the nucleic acid of the vaccine is introduced in the form of a vector wherein expression is under control of a viral promoter. For more detailed discussions of vectors according to the invention, cf. the discussion below. Also, detailed disclosures relating to the formulation and use of nucleic acid vaccines are available, cf. Donnelly JJ et al, 1997, Annu. Rev. Immunol. 15: 617-648 and Donnelly JJ et al., 1997, Life Sciences 60: 163-172. Both of these references are incorporated by reference herein.

An important part of the invention pertains to a novel method for selecting an appropriate immunogenic analogue of a cell-associated polypeptide antigen which is weakly immunogenic or non-immunogenic in an animal, said immunogenic analogue being capable of inducing a CTL response in the animal against cells displaying an MHC Class I molecule bound to an epitope derived from the cell-associated polypeptide antigen. This novel method comprises the steps of

a) identifying at least one subsequence of the amino acid sequence of the cell-associated polypeptide antigen which does not contain known or predicted CTL epitopes,

b) preparing at least one putatively immunogenic analogue of the cell-associated polypeptide antigen by introducing, in the amino acid sequence of the cell-associated polypeptide antigen, at least one T_H epitope foreign to the animal in a

position within the at least one subsequence identified in step a), and

c) selecting the/those analogues prepared in step b) which are verifiably capable of inducing a CTL response in the animal.

Alternatively, the above selection method involves the preparation of a nucleic acid fragment for nucleic acid vaccination purposes. In that situation, it is required that the encoded peptide includes at least one T_H epitope.

When the analogue is derived from an antigen which is exposed to the extracellular phase, it is preferred that the subsequence identified in step a) further does not contain cysteine residues, or, alternatively, that the T_H epitope introduced in step b) does not substantially alter the pattern of cysteine residues. This approach facilitates the preservation of spatial B-cell epitopes in the resulting construct which are similar to the B-cell epitopes in the weak, cell-associated polypeptide antigen.

For the same reasons it is preferred the subsequence identified in step a) further does not contain known or predicted glycosylation sites, or, alternatively, wherein the T_H epitope introduced in step b) does not substantially alter the glycosylation pattern.

Certain of the weak, cell-associated polypeptide antigens exert undesired effects by having a pathophysiological role. It is desired that these effects are not exerted by the vaccination constructs, and therefore it is preferred that the subsequence identified in step a) contributes significantly to a pathophysiological effect exerted by the cell-associated polypeptide antigen, and that the introduction in step b) of

the foreign T_H epitope reduces or abolishes said pathophysiological effect.

Another important consideration pertains to the question of immunological cross-reactivity of the vaccine's polypeptide product with other non-pathological self-proteins. Such cross-reactivity should preferably be avoided and hence an important embodiment of this method of the invention is one where the subsequence identified in step a) is homologous to an amino acid sequence of a different protein antigen of the animal, and where the introduction of the T_H epitope in step b) substantially removes the homology.

Related to this embodiment is an embodiment where any amino acid sequences which 1) are not normally exposed to the extracellular phase and 2) which may constitute B-cell epitopes of the weak, cell-associated polypeptide antigen, are not preserved in the analogue. This can be achieved by exchanging such amino acid sequences with T_H epitopes which do not constitute B-cell epitopes, by completely removing them, or by partly removing them.

On the other hand, it is preferred that any "true" B-cell epitopes of the weak cell-associated polypeptide antigen are preserved to a high degree, and therefore an important embodiment of the selection method of the invention involves that the introduction in step b) of the foreign T_H epitope results in preservation of a substantial fraction of B-cell epitopes of the cell-associated polypeptide antigen. It is especially preferred that the analogue preserves the overall tertiary structure of the cell-associated polypeptide antigen.

The preparation in step b) is preferably accomplished by molecular biological means or by means of solid or liquid phase peptide synthesis. Shorter peptides are preferably

prepared by means of the well-known techniques of solid- or liquid-phase peptide synthesis. However, recent advances in this technology has rendered possible the production of full-length polypeptides and proteins by these means, and therefore it is also within the scope of the present invention to prepare the long constructs by synthetic means.

After having identified the useful analogues according to the above-discussed method, it is necessary to produce the analogue in larger scale. The polypeptides are prepared according to methods well-known in the art.

This can also be done by molecular biological means. This comprises a first step of preparing a transformed cell by introducing, into a vector, a nucleic acid sequence encoding an analogue which has been selected according to the method and transforming a suitable host cell with the vector. The next step is to culture the transformed cell under conditions facilitating the expression of the nucleic acid fragment encoding the analogue of the cell-associated antigen, and subsequently recovering the analogue from the culture supernatant or directly from the cells, e.g. in the form of a lysate). Alternatively, the analogue can be prepared by large-scale solid or liquid phase peptide synthesis, cf. above.

Finally, the product can, depending on the cell chosen as a host cell or the synthesis method used, be subjected to artificial post-translational modifications. These can be refolding schemes known in the art, treatment with enzymes (in order to obtain glycosylation or removal of undesired fusion partners, chemical modifications (again glycosylation is a possibility), and conjugation, e.g. to traditionally carrier molecules.

Specific exemplary targets for the method of the invention

As discussed above, preferred weak, cell-associated polypeptide antigens are tumour-associated antigens. A non-limiting list of these consists of prostate-specific membrane antigen (PSM), Her2, fibroblast growth factor, e.g. FGF-8 such as FGF-8A and FGF-8b, human chorionic gonadotropin (hCG), BAGE, beta-actinin, Carcino Embryonic Antigen (CEA), Cathepsins, CD33, CDK-4, E6, E7, EGFR, EGP40 (KSA), GAGE, Gastrin-releasing peptide (bombesin), GnTV, GP1, gp100 (melanoma-associated), gp75 (melanoma-associated), IGFR1, K3, MAGE such as MAGE1 and MAGE3, MART, Matrix Metalloproteinases such as MMP2, MMP3, MMP7, and MMP9, aberrantly glycosylated Mucin, such as MUC-1, MUC-2, MUC-3, and MUC-4, MUM-1, p15 (melanoma-associated), PAI-1, PDGF, Plasminogen such as uPA, RAGE, TGF-α, TRP-1/gp-75, TRP-2, Tyrosinase, and ZAG.

In the following, 3 specific tumour-associated antigens will be discussed in detail.

Prostate-specific membrane-associated antigen, PSM

In U.S.A., prostate cancer is the second leading cause of cancer death (app. 40,000 per year), and 200,000 patients per year are diagnosed (Boring 1993). Approximately 1 out of 11 men eventually will develop prostatic cancer. Furthermore, approximately 40-60% of prostate cancer patients eventually develop extraprostatic extension of the disease (Babaian 1994). The main strategy in the present invention is to use a therapeutic vaccine as a supplementary therapy to prostatectomy in order to eliminate residual tumour tissue and metastases.

Several pathologic conditions are located to the prostate gland, including benign growth (BPH), infection (prostatitis) and neoplasia (prostatic cancer).

The biological aggressiveness of prostatic cancer is variable. In some patients the detected tumour remains a latent histologic tumour and never becomes clinically significant. In other patients, the tumour progresses rapidly, metastasises and kills the patient in a relatively short time period (2-5 years).

The current primary treatment of prostate cancer is prostatectomy. However, due to the extensive spreading of prostate cancer cells the majority of prostatic cancer patients are not cured by local surgery. Patients with non-confined disease eventually receive systemic androgen ablation therapy, but the annual death rate from prostatic cancer has not declined at all over the 50 years since androgen ablation became standard therapy for metastatic disease.

PSM is a membrane protein which is highly specific for prostatic tissues, benign as well as malignant. Therefore, if surgery was successful, prostatectomised cancer patients should theoretically no longer express PSM except on residual malignant prostate tumour tissue or metastases originating from the tumour. In other words, in these patients PSM is a true tumour specific antigen. By inducing a strong CTL response and/or a strong polyclonal antibody response towards PSM, it is expected that residual tumour tissue can be eliminated.

Interestingly, upregulation of PSM expression is seen following androgen-deprivation therapy of prostate cancer patients (Wright 1996). This would make a PSM-targeted treatment very well-suited to follow the traditional androgen-deprivation therapy.

PSM was first identified in 1987 as a result of generating a monoclonal antibody, 7E11-C5.3, raised against an isolated

prostatic cancer cell, LNCaP (Horoszewicz 1987). The antibody recognised both normal and malignant prostatic epithelium, and was used in 1993 to purify and microsequence the PSM protein and eventually clone the gene (Israeli 1993).

PSM is a type II transmembrane glycoprotein with a molecular weight of 84 kD as predicted from the nucleic acid sequence whereas the glycosylated version has an observed molecular weight of 100 kD (Israeli 1993). Sequencing of the gene encoding PSM revealed a putative membrane spanning region in connection with three cytosolic arginine anchor residues. The extracellular part of PSM constitute 707 of the total 750 amino acids of the protein, whereas the cytoplasmic domain is predicted to be 19 amino acids long (Israeli 1993). PSM-specific mRNA has been detected in prostate tumour tissue (Israeli 1994), indicating that the tumour antigen is not an aberrantly glycosylated protein which is the case with e.g. the Tn- or sTn-tumour antigens.

The full length PSM cDNA has been transfected into and expressed in a PSM negative human prostate cancer cell line, PC-3 (Kahn 1994). Furthermore, the full length (2.65 kilobases) cDNA has been transcribed and translated *in vitro* (Kahn 1994).

It has recently been demonstrated that PSM possesses hydrolytic activity resembling that of the N-acetylated α-linked acidic dipeptidase (NAALADase). NAALADase is a membrane-bound hydrolase of the nervous system, which catabolises the neuropeptide N-acetylaspartyl glutamate (NAAG) in order to affect the glutamatergic signalling processes. It has been shown that PSM possess NAAG-hydrolysing activity and that this activity can be inhibited by the NAALADase inhibitors quisqualic acid and β-NAAG (Carter 1996). It is

still not known whether this activity of PSM has any relevant biological function.

It is of significant importance to predict whether undesired cross-reactivity with other proteins accessible for antibodies would be expected following treatment with an autovaccine inducing PSM-specific autoantibodies. It has been shown that a part of the coding region of the PSM gene (amino acids positions 418-567) has 54% homology to the human transferrin receptor (Israeli 1993). Also, sequence homology with the NAALADase enzyme has been found. A 1428 bp partial cDNA from a rat brain library isolated using a rat anti-NAALADase antiserum showed 86% sequence identity with 1428 bases of the human PSM cDNA (Carter 1996). However, no identification of a functionally relevant similarity with other known peptidases could be made.

The homology to the transferrin receptor is very low and will preferably be disrupted in some of the inventive constructs. The observed partial homology with a rat brain protein as an obstacle for a PSM-vaccine, partly because of the inability of antibodies to penetrate the blood-brain barrier. Altogether, even with the most PSM-like construct, it is not expected to experience cross-reactivity with other proteins in the patients.

From earlier studies it is clear that PSM is expressed on all prostate cancer cells and prostate originating metastases tested. All other cancers tested, such as carcinomas, sarcomas and melanomas of different tissues as well as a large panel of non-prostatic human cancer cell lines have proven PSM negative.

In addition to this, a very large number of other tissues have been found to be PSM negative. These include colon, breast,

lung, ovary, liver, urinary bladder, seminal vesicles, testis, uterus, bronchus, spleen, pancreas, tongue, esophagus, stomach, thyroid, parathyroid, adrenal, lymph node, aorta, vena cava, skin, mammary gland and placenta.

Although PSM is predominantly found as a membrane bound molecule on prostate tissue small amounts of PSM can also be detected in the sera of normal individuals and in elevated levels in prostate cancer patients (Rochon 1994, Murphy 1995). The level of circulating PSM in these patients therefore allows a serological monitoring of the effectiveness of a PSM vaccine.

In conclusion, based on the entire amount of data available to date, PSM is a true prostate-specific antigen. This means that in patients who have undergone prostatectomy, PSM is a tumour specific self-antigen. An effective PSM vaccine is therefore likely to target only prostatic or prostate-originating metastatic tissue.

As will be clear from Example 1 the method of the invention is preferably entails that foreign T_H-cell epitope is introduced in a part of the PSM amino acid sequence defined by SEQ ID NO: 2 positions 16-52 and/or 87-108 and/or 210-230 and/or 269-289 and/or 298-324 and/or 442-465 and/or 488-514 and/or 598-630 and/or 643-662 and/or 672-699. Furthermore, a modified PSM molecule which has a foreign T_H-epitope introduced in these positions is also a part of the invention.

Accordingly, the invention also pertains to an analogue of human PSM which is immunogenic in humans, said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of PSM and including at least one foreign T_H epitope as discussed herein. Preferred PSM analogues are those wherein the at least one foreign T_H epitope is present as

an insertion in the PSM amino acid sequence or as a substitution of part of the PSM amino acid sequence or as the result of deletion of part of the PSM amino acid sequence, and most preferred analogues are those wherein a foreign T_H-cell epitope is introduced in a part of the PSM amino acid sequence defined by SEQ ID NO: 2 positions 16-52 and/or 87-108 and/or 210-230 and/or 269-289 and/or 298-324 and/or 442-465 and/or 488-514 and/or 598-630 and/or 643-662 and/or 672-699.

Human Chorionic Gonadotropin (HCG)

The relationship between embryonic markers and malignant phenotypes has been under discussion for many decades. An increasing body of data suggests that at least one such marker, human chorionic gonadotropin beta (hCGβ), is consistently detected on cancer cells of many different histological origins, and that expression of this protein often correlates with increased metastatic properties. A humoral immune response directed against this soluble protein may reduce the chances of tumour spreading and/or may inhibit the recurrence of new primary growths post-surgery.

Human chorionic gonadotropin belongs to a family of glycoprotein hormones, including follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and luteinizing hormone (LH), all of which are important regulators of reproductive expression and fetal survival. The members of this family of hormones are heterodimers, which share a common α-chain. The β-chain is unique to each hormone and provides the specificity, with the β-chain of LH exhibiting the strongest sequence homology to hCGβ (approximately 80%). The apparent molecular weight of hCG-holo, is 37 kD, of which one third is contributed by carbohydrate. The post-translational sugar modifications include both N-linked and O-linked carbohydrate. Abundant sialic acid residues are present and

these give the protein a large negative charge. The crystal structure of hCG-holo has been solved (Lapthorn *et al.*, 1994).

Based on the crystal structure it was found that hCG exhibits homology to a family of growth factors, including PDGF and TGFβ (Lapthorn *et al.*, 1994). This suggests that hCG expression may help regulating cancer cell growth.

Human chorionic gonadotropin is a glycoprotein hormone, which is produced by the placental syncytiotrophoblasts soon after conception, and it is essential for successful gestation in the pregnant woman.

The pathophysiological role of embryonic markers for the development or maintenance of a cancer mass is not known. However, it is of interest to note that trophoblasts (where these proteins are normally produced) have both *angiogenic* and *invasive* characteristics, both of which are also necessary properties for a cancer cell. Further, it has been suggested that hCG (or its subunits) can inhibit maternal cellular immune responses to fetal tissue. For example, studies have shown that hCG directly suppresses T cell responses (Jacoby *et al.*, 1984) and it has been proposed that because the lymph nodes draining (in this case) a primary melanoma tumour, are immunosuppressed, a more favourable environment for metastatic tumours to establish themselves may result. As a consequence, expression of hCGβ may help cancer cells spread into the secondary lymphoid organs. Finally, as mentioned above, structural homology between hCG and a number of growth factors have been demonstrated. Another possibility is therefore that secretion of hCG by cancer cells may give the tumour a growth advantage.

Expression of hCGβ has been shown in many different types of cancer, for example: a) Prostate adenocarcinoma: positivity

for hCGβ on tissue sections were seen for patients with poor prognosis, irrespective of the histological grade of the tumour (Sheaff *et al.*, 1996), b) different kinds of lung carcinomas; squamous cell (SQCC), adenocarcinoma (AC), and large cell (LCC), all showed a high percentage of reactivity for hCGβ (Boucher *et al.* 1995), c) pancreatic adenocarcinoma (Syrigos *et al.*, 1998), d) neuroblastomas, brain cancers, retinoblastomas (Acevedo et al., 1997), e) malignant melanoma (Doi *et al.*, 1996), f) bladder carcinomas (Lazar *et al.*, 1995). A recent paper describes a DNA approach, in which mice were immunized with a hCGβ expression construct (Geissler *et al.*, 1997). In this *in vivo* model inhibition of tumour growth was strongly associated with CTL-activity, however high titers of antibodies (which neutralized the biological effect of intact hCG on its cellular receptor) were also detected.

The use of hCG as an immunogen has been described in several papers, focussing on its use as a contraceptive vaccine (Talwar *et al.*, 1976 and Talwar *et al.*, 1994). A very high degree of efficacy and safety has been observed in an anti-fertility clinical trial, using a vaccine against hCG-holo (Talwar *et al.*, 1994). Phase I clinical trials of cancer patients with a vaccine against a synthetic carboxy-terminal peptide of hCGβ conjugated to diphteria toxoid have also been conducted (Triozzi *et al.*, 1994) and phase II trials are underway. Despite the fact that the idea to use hCGβ as a cancer vaccine target has been around for some time, it has not been explored in conjunction with the AutoVac technology.

It is known that cells from non-embryonic tissue, or benign neoplasms, do not express hCGβ. Therefore, there should be no potential side effects from vaccination against this molecule (apart from the effects on pregnancy). Because it is expressed by so many different kinds of cancers this molecule has been proposed to be the "definitive cancer biomarker" (Acevedo *et*

al., 1995 and Regelson W., 1995) and as such would be an attractive target to go after.

Suitable animal models for Further studies of the efficacy of a hCG based vaccine can be found in Acevedo *et al.*, Cancer Det. and Prev. Suppl. (1987) 1: 477-486, and in Kellen *et al.*, Cancer Immunol. Immun.Ther. (1982) 13: 2-4.

Her-2

The tyrosine kinase receptors Her-2 and EGFr are believed to play a crucial role in the malignant transformation of normal cells and in the continued growth of cancer cells. Overexpression is usually linked to a very poor prognosis. During the past few years there has been many reports concerning the use of antibodies against these receptors as therapy for cancers that overexpress either or both of these receptors. Genentech Inc. has finished several successful clinical trials on breast cancer patients using a monoclonal antibody against Her-2 and has recently obtained an FDA approval for the marketing of the anti-Her-2 monoclonal antibody preparation, Herceptin®.

The autovacination technology disclosed herein as applied on the Her-2 molecule would elicit polyclonal antibodies that would predominantly react with Her-2. Such antibodies are expected to attack and eliminate tumour cells as well as prevent metastatic cells from developing into metastases. The effector mechanism of this anti-tumour effect would be mediated via complement and antibody dependent cellular cytotoxicity.

Dependent on the choice of constructs, the induced autoantibodies could also inhibit cancer cell growth through inhibition of growth factor dependent oligo-dimerisation and

internalisation of the receptors. And, most importantly, the Her-2 analogues are expected to be able to induce CTL responses directed against known and/or predicted Her-2 epitopes displayed by the tumour cells

Her-2 is a member of the epidermal growth factor receptor family (c-erbB) which consists of four different receptors to date: c-erbB-1 (EGFr), c-erbB-2 (Her-2, c-Neu), c-erbB-3 and c-erbB-4 (*Salomon et al, 1995*). C-erbB-3 and c-erbB-4 are less well characterised than EGFr and Her-2. Her-2 is an integral glycoprotein. The mature protein has a molecular weight of 185 kD with structural features that closely resembles the EGFr receptor (*Prigent et al, 1992*). EGFr is also an integral membrane receptor consisting of one subunit. It has an apparent molecular weight of 170 kD and consists of a surface ligand-binding domain of 621 amino acids, a single hydrophobic transmembrane domain of 23 amino acids, and a highly conserved cytoplasmic tyrosine kinase domain of 542 amino acids. The protein is N-glycosylated (Prigent et al, 1994).

All proteins in this family are tyrosine kinases. Interaction with the ligand leads to receptor dimerisation, which increases the catalytic action of the tyrosine kinase (*Bernard. 1995, Chantry 1995*). The proteins within the family are able to homo- and heterodimerise which is important for their activity. The EGFr conveys growth promoting effects and stimulates uptake of glucose and amino acids by cells (*Prigent et al 1992*). Her-2 also conveys growth promoting signals. Only EGFr binds EGF and TGF-alpha. These ligands do not bind to the other receptors in the family (*Prigent et al., 1992*). The ligands for Her-2 are not fully determined. However, heregulin has been shown to induce phosphorylation by activating Her-2. This does not appear to be due to a direct binding to the receptor but it is believed that heregulin is a ligand for

erbB-3 and erbB-4 which then activates Her-2 by oligo-dimerisation (*Solomon et al 1995*).

The homology between the proteins of EGF receptor family is most pronounced in the tyrosine kinase domain at the cytoplasmic part of the molecules (82% between EGFr and Her-2). The homology is less in the extracellular part - from 41% to 46% in different domains (*Prigent et al, 1992*).

The epidermal growth factor receptor is expressed on normal tissues in low amounts, but it is overexpressed in many types of cancers. EGFr is overexpressed in breast cancers (*Earp et al, 1993, Eppenberger 1994*), gliomas (*Schlegel et al, 1994*), gastric cancer (*Tkunaga et al, 1995*), cutaneous squamous carcinoma (*Fujii 1995*), ovarian cancer (*van Dam et al, 1994*) and others. Her-2 is also expressed on few normal human tissues in low amount, most characteristically on secretory epithelia. Over expression of Her-2 occurs in about 30% of breast, gastric, pancreatic, bladder and ovarian cancers.

The expression of these receptors varies depending on the degree of differentiation of the tumours and the cancer type, e.g., in breast cancer, primary tumours overexpress both receptors; whereas in gastric cancer, the overexpression occurs at a later stage in metastatic tumours (*Salomon et al, 1995*). The number of overexpressed receptors on carcinoma cells is greater than 10^6/cell for several head and neck cancers, vulva, breast and ovarian cancer lines isolated from patients (*Dean et al, 1994*).

There are several reasons why the EGFr family of receptors constitute suitable targets for tumour immunotherapy. First, they are overexpressed in many types of cancers, which should direct the immune response towards the tumour. Second, the tumours often express or overexpress the ligands for this

family of receptors and some are hypersensitive to the proliferative effects mediated by the ligands. Third, patients with tumours that overexpress growth factor receptors often have a poor prognosis. The overexpression has been closely linked with poor prognosis especially in breast cancer, lung cancer and bladder cancer (2) and is apparently associated with invasive/metastatic phenotypes, which are rather insensitive to conventional therapies (*Eccles et al, 1994*).

Overexpression of Her-2 is in some cases a result of amplification of the gene and in other cases increased transcription and translation. The overexpression of Her-2 is associated with poor prognosis in breast, ovarian cancers, gastric cancer, bladder cancer and possibly in non-small cell lung cancers (*Solomon et al, 1995*).

Phase I clinical trials have been performed with a bispecific antibody in patients with advanced breast and ovarian cancer. The antibody was bispecific against Her-2 and FcγRI (*Weiner et al, 1995*). Efficient lysis of Her-2 over expressing tumour cells was observed with a bispecific antibody against Her-2 and CD3 (*Zhu et al, 1995*).

Treatment of scid mice xenografted with human gastric cancer with an anti-Her-2 monoclonal antibody prolonged the survival of the mice (*Ohniski et al, 1995*). The anti-tumour activities of monoclonal antibodies against Her-2, *in vitro* and *in vivo* is not due to an identical mechanism; they may act as partial ligand agonists, alter Her-2 receptor turnover and phosphorylation or may affect dimerization (*Lupu et al, 1995*).

Similarly, it has been shown that antibodies to EGFr can also interfere with growth factor interactions. (Baselga *et al*, 1994, Modjahedi *et al*, 1993a, Wu *et al*, 1995, Modjahedi *et al*,

1993b, Tosi *et al*, 1995, Dean *et al*, 1994, Bier *et al*, 1995, Modjtahedi *et al*, 1996, Valone 1995).

Hence, an important embodiment of the methods of the invention is one wherein the foreign T-cell epitope is introduced in a part of the Her-2 amino acid sequence defined by SEQ ID NO: 4 positions 61-75 and/or 105-119 and/or 151-165 and/or 212-226 and/or 252-266 and/or 327-341 and/or 371-385) and/or 467-481 and/or 581-595 and/or 72-86 and/or 146-160 and/or 221-235 and/or 257-271 and/or 387-401, cf. the Examples.

Accordingly, the invention also relates to an analogue of human Her2 which is immunogenic in humans, said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of Her2 and including at least one foreign T_H epitope as discussed herein. It is preferred that the at least one foreign T_H epitope is present as an insertion in the Her2 amino acid sequence or as a substitution of part of the Her2 amino acid sequence or as the result of deletion of part of the Her2 amino acid sequence. Most preferred analogues are those defined above, *i.e.* those wherein the foreign T-cell epitope is introduced in a part of the Her-2 amino acid sequence defined by SEQ ID NO: 4 positions 61-75 and/or 105-119 and/or 151-165 and/or 212-226 and/or 252-266 and/or 327-341 and/or 371-385) and/or 467-481 and/or 581-595 and/or 72-86 and/or 146-160 and/or 221-235 and/or 257-271 and/or 387-401.

FGF8b

It has been shown by several investigators that FGF8b can induce proliferation, transformation, differentiation and in some cases greatly increase the tumorigenicity of mammalian cells and tissues (Tanaka 1992, Kouhara 1994, Lorenzi 1995, MacArthur 1995a, Crossley 1996a, 1996b, Ghosh 1996, Ohuchi 1997a, Rudra-Ganguly 1998). These effects are primarily

mediated through the binding of FGF8b to members of the fibroblast growth factor receptors FGFR2, FGFR3, and FGFR4 (MacArthur 1995b, Blunt 1997, Tanaka 1998). Thus, cells expressing one of these receptors and FGF8b will provide an autocrine growth-signalling cascade for themselves. We therefore believe that an autovaccine against FGF8b will be a very efficient means of treating FGF8-expressing cancer types.

Prostate cancer

The biological aggressiveness of prostatic cancer is variable. In some patients the detected tumor remains a latent histologic tumor and never becomes clinically significant. In other patients, the tumor progresses rapidly, metastasizes, and kills the patient in a relatively short time (2-5 years).

For the purpose of diagnosis, and to follow the response to therapy of prostate cancer, determination of the circulating levels of two proteins has primarily been used: prostatic acid phosphatase (PAP) and prostate specific antigen (PSA) (Nguyen 1990, Henttu 1989). Due to disrupture of the normal architecture of the prostate gland in response to cancer development, these soluble proteins are released into the circulation where they can be detected as markers for e.g. metastatic spread.

The current primary treatment of prostate cancer is prostatectomy. However, due to the extensive spreading of prostate cancer cells the majority of prostatic cancer patients are not cured by local surgery. Patients with non-confined disease receive systemic androgen ablation therapy, but the annual death rate from prostatic cancer has not declined at all over the 50 years since androgen ablation became standard therapy for metastatic disease.

RT-PCR analysis has shown that FGF8 mRNA is produced by the human prostatic epithelial tumor cell lines LNCaP, PC-3, ALVA-31, and DU145 respectively, with FGF8b being the most prominent isoform (Tanaka 1995, Ghosh 1996). The growth of the androgen-responsive LNCaP cells are stimulated by addition of recombinant FGF8b (Tanaka 1995), while DU145 cells could be growth inhibited by transfection with vira expressing anti-sense FGF8b (Rudra-Ganguly 1998). This, together with evidence from developmental studies discussed below, indicate a role for FGF8b in maintaining the cancerous state of these cell lines.

Using FGF8a cDNA for in situ hybridization experiments, Leung and co-workers have shown that a high proportion (71% (n=31)) of prostatic cancers produce FGF8 mRNA, and that the amount of FGF8 mRNA correlate with the severeness of the tumors ($P<0.05$) (Leung 1996). Immunohistochemical analysis a monoclonal antibody against FGF8, has detected the protein in 93% (n=43) of human prostate cancers (Tanaka 1998). Normal prostatic tissue or benign prostatic hyperplasia does not produce FGF8 mRNA, or contain FGF8 protein (Leung 1996, Yoshimura 1996, Ghosh 1996, Tanaka 1998).

These results indicate that an autovaccine against FGF8b would be reactive against prostatic tumor tissue and thus, extremely valuable in the treatment of prostatic cancer.

Breast cancer

The current treatment of breast cancer is surgery. However, due to the extensive spreading of breast cancer cells a large part of breast cancer patients are not cured by local surgery. Patients with non-confined disease eventually receive androgen ablation therapy, chemotherapy, and or radiation therapy. The

annual death rate from breast cancer is, however, still relatively high.

FGF8 was originally isolated from a mouse mammary carcinoma cell-line (SC-3), from which the expression could be induced by adding androgen to the medium (Nonomura 1990). The protein is also known to induce the proliferation of these as well as other mammalian cells. Recently FGF8b mRNA has been shown to be present in eight (n=8) human breast cancer cell lines (MDA-MB-231, MDA-MB-415, ZR 75-1, T-47-D, SK-BR-III, PMC-42, HBL-100 and MCF-7) (Tanaka 1995, Payson 1996, Wu 1997, Marsh 1998).

Wnt-1 transgenic mice infected with mouse mammary tumor virus (MMTV) develop mammary tumors. FGF8 transcription is activated in 50% of these tumors (MacArthur 1995c, Kapoun 1997).

Transgenic mice that are carrying the FGF8b cDNA under control of the very specific mouse mammary tumor virus (MMTV) promoter, are shown to spontaneously develop FGF8b expressing mammary tumors (Coombes, personal communication).

Very recent data strongly indicates that FGF8b expression is related to breast cancer (Tanaka 1998, Marsh 1998). Tanaka and co-workers utilized a new monoclonal FGF8 antibody in immunohistochemical studies. They showed that FGF8 was present in 67% (n=12) of breast cancers, and that androgen receptors were present in 89% of breast diseases (Tanaka 1998). Using a semi-quantitative RT-PCR method it was shown that elevated levels of FGF8 mRNA were found in malignant compared to non-malignant breast tissues. Significantly more malignant tissues are expressing FGF8 ($p=0.019$) at significantly higher levels ($p=0.031$) (68 breast cancers and 24 non-malignant breast tissues) (Marsh 1998). This is in contrast to a study

where FGF8b mRNA was found in only one of nine breast cancers (Wu 1997).

Although it has not yet been established that FGF8b functions as an autocrine growth factor, these findings in our opinion suggest that an autovaccine against FGF8b would be reactive against a large percentage of breast and prostate cancers. The data presented by Marsh, and Tanaka indicate that an autovaccine against FGF8b could be used for treatment of both breast and prostate cancer. A significant proportion of these cancers could, in agreement with the general opinion, be dependent of FGF8b in maintaining the cancerous state of the cells.

Description of FGF8b

FGF8 belongs to the family of fibroblast growth factors (FGFs). These growth regulatory proteins are small ~200 amino acid residue proteins that all are involved in the induction of proliferation and differentiation of a wide range of cells. For a recent review of the involvement of the fibroblast growth factors in vertebrate limb development, see Johnson 1997. The FGF family members are evolutionary related and share 20-50% amino acid sequence identity.

FGF8b is a splice variant of FGF8, originally termed androgen induced growth factor (AIGF). AIGF was first identified as a protein secreted by a murine mammary carcinoma derived cell line (SC-3) upon stimulation with androgen (Nonomura 1990). The murine FGF8 gene contains 6 exons, potentially coding for eight different FGF8-isoforms (FGF8a-h), differing only in the N-terminal part of the molecules (Crossley 1995, MacArthur 1995b). Human FGF8 has the same gene structure as the murine gene. However, due to a stop codon in exon 1B, human FGF8 can possibly exist in four different isoforms namely FGF8a, FGF8b,

FGF8e, and FGF8f (Gemel 1996). The gene structures and the amino acid sequences of the four human isoforms are illustrated in Fig. 5.

Mature FGF8b contains 193 amino acid residues, and has a calculated molecular weight of 22.5kDa. The highly basic protein contains 21 arginine and 14 lysine residues resulting in a calculated isoelectric point of 10.84, and a calculated positive charge of 19,8 at pH 7.0. It contains two cysteine residues, and has two potential N-glycosylation sites. Due to the nature of the investigations performed involving FGF8b very little is known about the FGF8b protein moiety. It has, however, been expressed heterologously from bacteria, purified by the use of a C-terminal hexa-Histidine tag, and *in vitro* refolded to a soluble and biologically active state (MacArthur 1995a, Blunt 1997).

Biological activity of FGF8b

As mentioned earlier, FGF8b was first isolated as a factor that was released from a mouse mammary tumour cell line, and it has been shown that this protein can induce the proliferation of these cells. The morphological changes mimic those induced by testosterone, which is also know to induce the synthesis of FGF8b mRNA (Tanaka 1992). The proliferation can be inhibited by FGF8b antisense oligos (Nonomura 1990, Tanaka 1992, Yamanishi 1994). Indeed, a human prostate cancer cell line DU145 could be growth inhibited by transfection with vira expressing anti-sense FGF8b (Rudra-Ganguly 1998).

FGF8b has by several investigators been shown very efficient in inducing the transformation of NIH3T3 cells (Kouhara 1994, Lorenzi 1995, MacArthur 1995a). By using recombinantly expressed proteins, it was shown that this induction of morphological changes is far more efficient with FGF8b than

when using FGF8a or FGF8c (MacArthur 1995a, Ghosh 1996). Recently, it was shown that the N-terminal half of the FGF8b molecule is sufficient for transformation of NIH3T3 cells, and that even the small FGF8b specific peptide (QVTVQSSPNFT) could enable the cells to grow 2-3 times longer than normal in 0.1% serum (Rudra-Ganguly 1998). Interestingly, NIH3T3 cells stably transfected with an expression vector encoding FGF8b is very tumorigenic when injected intraocularly into nude mice (Kouhara 1994, Ghosh 1996).

in vivo, FGF8b is known to be expressed at certain stages of development in vertebrates. A summary of the biological roles assigned to FGF8b is shown in the following table. An excellent review of various models for vertebrate limb development is given by Johnson 1997.

Table: Various sites/tissues known to produce FGF8, and the proposed biological role(s).

Action/mechanism/presence (species)	References
Present in the developing limb buds (mouse)	Heikinheimo 1994, Ohuchi 1994
Limb bud outgrowth (chicken)	Kuwana 1997, Xu 1998
Induction of ectopic limb formation from mesoderm (chicken)	Crossley 1996b
Induction of midbrain formation from the caudal diencephalon (chicken)	Crossley 1996a
Initiation of wing outgrowth in a wingless mutant (chicken)	Ohuchi 1997a
Role in dorsoventral patterning of the gastrula (zebrafish)	Fürthauer 1997

Required during gastrulation, cardiac, craniofacial, forebrain, midbrain and cerebellar development (tissue specific knockout mice)	Meyers 1998
Role in tooth morphogenesis (mouse)	Kettunen 1998

It is believed that FGF8b mediates its action through binding to the fibroblast growth factor receptors (FGFR's). Specifically FGF8b is known to be able to activate FGFR2c, FGFR3c, FGFR4c, and to some extent also FGFR1c, but not FGFR1b, -2b or -3b (MacArthur 1995b, Blunt 1997). In case of the induction of outgrowth of ectopic chicken limbs, it is strongly suggested that FGF10, FGFR2, and FGF8 are interacting and that this is sufficient for outgrowth (Kuwana 1997, Xu 1998). These receptor-data are supporting the hypothesis that FGF8b acts in an auto- and paracrine manner, leading to the normal outgrowth and patterning of several anatomical structures during vertebrate development. Importantly, FGF8 "total knock out" mice do not survive probably due to the elaborate involvement of the protein in the development of the embryo.

Homology to other proteins

It is of significant importance to predict whether undesired cross-reactivity with other proteins accessible for antibodies would be expected following treatment with an autovaccine inducing FGF8b specific autoantibodies. Due to the high degree of sequence identity between FGF8b and the other FGF8 molecules, an autovaccine will be expected to cross-react with these proteins. This, however, will presumably be advantageous, since none of these proteins are reported to be expressed in tissues or by cell-lines that do not already express FGF8b.

Amino acid residues 55 through 175 of FGF8b shows a relatively low but significant degree of sequence identity to the other FGFs. It is commonly accepted (and several times proven) that a significant degree of sequence identity between two protein domains is also reflected in a high degree of tertiary structure similarity. Therefore, the FGF family members are all generally expected to be structurally similar. The three dimensional structure of FGF2 has been resolved from crystals as well as in solution (Ago 1991, Zhang 1991, Zhu 1991, Eriksson 1993, Blaber 1996, Moy 1996). FGF2 is composed entirely of beta-sheet structure, comprising a three-fold repeat of a four-stranded antiparallel beta-meander. This beta-barrel structure is totally conserved between interleukin 1, FGF2 (or basic FGF), and FGF1 (or acidic FGF). Nuclear magnetic resonance analysis of FGF2 in solution has shown that the N-terminal part of the molecule forms a relatively flexible structure. The remaining part of FGF8b (amino acid residues 1-54 and 176-215) only shows a low degree of sequence identity to known proteins.

Based on the structural and alignment data, it is generally assumed that the three dimensional structural core of the other fibroblast growth factors closely resemble those of FGF1 and FGF2. These structural considerations are important factors in our design of the FGF8b mutant autovaccine molecules.

Importantly, due to the relatively low degree of sequence identity between FGF8 and any of the other members of the FGF family, the surface of FGF8 would be very different from that of other FGFs, thereby minimizing the cross-reactivity of FGF8b autovaccine generated antibodies with other FGF family members. Due to the very low degree of homology to other proteins than the fibroblast growth factors, we do not expect

an autovaccine against FGF8b to cross-react with any other proteins.

It should be emphasized, however, that an autovaccine against FGF8b probably would cross react with all isoforms of FGF8. This will, however, presumably not be a problem since none of the FGF8 isoforms are expected to be expressed at significant levels in the adult. It is even possible that this cross reaction will be beneficial in the treatment of cancer, since it has been shown that at least some cancer cell lines express other isoforms in addition to FGF8b.

Tissue distribution of FGF8b

Ideally, the induced autoantibodies and the subsequent effector mechanisms as well as the expected CTL response raised by autovaccination should only be directed towards tissues that are to be eliminated in the patient. Therefore, the tissue distribution of the antigen, which is targeted by an autovaccine, is an issue of great importance concerning the safety of the vaccine.

Table: Expression of FGF8b in various tissues and cells

Human	
Breast cancer cell lines (MDA-MB-231, MDA-MB-415, ZR 75-1, T-47-D, SK-BR-III, PMC-42, HBL-100, and MCF-7)	((RT-PCR) Tanaka 1995, Payson 1996, Wu 1997, Marsh 1998)
Breast tumors	((mAb) Tanaka 1998, (RT-PCR)M arsh 1998)
Normal breast tissue	((RT-PCR)Wu 1997, Marsh 1998 (mAb) Tanaka 1998)

Prostate cancer (93%)	((in situ hyb.) Leung 1996, (mAb) Tanaka 1998)
Breast disease	((mAb) Tanaka 1998)
Prostatic tumor cells (LNCaP, PC-3, DU145, and ALVA-31)	((RT-PCR) Tanaka 1995, Ghosh 1996)
fetal kidney	((Northern blot) Ghosh 1996)
adult prostate, testis, kidney	((RT-PCR) Ghosh 1996, Wu 1997)
teratocarcinoma cells (Tera-2)	((RT-PCR) Wu 1997)
Murine	
Breast cancer cell lines (SC-115, RENCA)	((RT-PCR) Yoshimura 1996)
Hypothalamus, Testis	((RT-PCR) Yoshimura 1996)
Mammary tumors (Wnt-1 transgenic)	((Northern blot) MacArthur 1995c)
Embryonic brain	((in situ hyb.) Crossley 1995, Heikinheimo 1994, Ohuchi 1994, Shimamura 1997, (RT-PCR) Blunt 1997)
Ovary, testis	((Northern blot) Valve 1997)
Developing face and limb buds	((pAb) MacArthur 1995b (in situ hyb.) Heikinheimo 1994, Ohuchi 1994, Crossley 1995)
Gastrula	((in situ hyb.) Crossley 1995)
Chicken	
Embryonic brain	((in situ hyb.) Crossley 1996a)
developing limb buds	((in situ hyb.) Ohuchi 1997a,b)

The above table shows a wide selection of tissue distribution, and cell line data of FGF8b expression. As seen from the

table, most of the data regarding tissue distribution is generated using the sensitive RT-PCR method. This is because Northern blotting analysis does not detect any FGF8b mRNA in any normal tissues except from fetal kidney. From this scarce data, it is generally assumed that expression of FGF8b mRNA in the adult is very limited, and thus, an autovaccine against FGF8b would presumably not be reactive against normal tissue. Due to the fact that small amounts of FGF8b could interact in unknown systems in the adult, the tissue distribution of the protein needs further analysis. There are, however, in our opinion no indications that an autovaccine against FGF8b would result in serious unwanted effects on the patients.

Effects of antibodies against FGF8b

So far, no attempts to treat prostate cancer using monoclonal antibodies have been published. Clinical trials with monoclonal antibodies are ongoing in breast cancer therapy studies, however.

Antibodies against FGF8b will probably block the interaction between FGF8b and its receptors, which will inhibit the cell membrane ruffling and cell proliferation, very likely decreasing the motility and invasiveness of the cancer cells.

Hence, the invention also relates to embodiments of the methods described herein where, where the foreign T-cell epitope is introduced in a part of the FGF8b amino acid sequence defined by SEQ ID NO: 6 positions 1-54 and/or 178-215 and/or 55-58 and/or 63-68 and/or 72-76 and/or 85-91 and/or 95-102 and/or 106-111 and/or 115-120 and/or 128-134 and/or 138-144 and/or 149-154 and/or 158-162 and/or 173-177. It should be noted that it is especially preferred *not* to introduce variations or modifications in positions 26-45 and in the C-terminus starting at amino acids 186-215, since these

stretches show the least homology with a recently discovered protein, FGF-18, which seems to be expressed in a variety of non-tumour tissues.

Accordingly, the invention also pertains to an analogue of human/murine FGF8b which is immunogenic in humans, said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of FGF8b and including at least one foreign T_H epitope as discussed herein. It is preferred that the at least one foreign T_H epitope is present as an insertion in the FGF8b amino acid sequence or as a substitution of part of the FGF8b amino acid sequence or as the result of deletion of part of the FGF8b amino acid sequence. Most preferred analogues in this embodiment are those where the foreign T-cell epitope is introduced in a part of the FGF8b amino acid sequence defined by SEQ ID NO: 6 positions 1-54 and/or 178-215 and/or 55-58 and/or 63-68 and/or 72-76 and/or 85-91 and/or 95-102 and/or 106-111 and/or 115-120 and/or 128-134 and/or 138-144 and/or 149-154 and/or 158-162 and/or 173-177.

Mucins

The invention also pertains to methods of the invention employing specifically modified versions of the human mucins, especially any of MUC-1 through MUC-4, preferably MUC-1. The analogues comprise the following structure

$$TR(-S-P-S-(TR)_m)_n$$

-where TR is a tandem repeat derived from the naturally occurring mucin, P is a foreign T_H-epitope as discussed herein, S is an inert spacer peptide having from 0 to 15 amino acid residues, preferably between 0 and 10 amino acid residues, and

n is an integer of from 1 to 30, and m is an integer from 1 to 10, preferably from 3 to 5.

When producing such a mucin analogue, the direct result will normally not have a glycosylation pattern as desired, *i.e.* an aberrant glycosylation pattern resembling that of a tumour derived mucin. However, it is possible to produce the analogue recombinantly in e.g. *E. coli* or by synthetic means, and subsequently glycosylating the product enzymatically so as to achieve a Tn or S-Tn glycosylation pattern. Alternatively, the polypeptide could be prepared in e.g. a CHO cell line lacking the relevant glycosylation enzyme.

It should be noted that preferred analogues of the invention comprise modifications which results in a polypeptide having a sequence identity of at least 70% with the polypeptide antigen or with a subsequence thereof of at least 10 amino acids in length. Higher sequence identities are preferred, e.g. at least 75% or even at least 80% or 85%. The sequence identity for proteins and nucleic acids can be calculated as $\left(N_{ref} - N_{dif}\right) \cdot 100 / N_{ref}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC (N_{dif}=2 and N_{ref}=8).

Nucleic acid fragments and vectors of the invention

It will be appreciated from the above disclosure that the analogues can be prepared by means of recombinant gene technology but also by means of chemical synthesis or semisynthesis; the latter two options are especially relevant when the modification consists in coupling to protein carriers (such as KLH, diphtheria toxoid, tetanus toxoid, and BSA) and

non-proteinaceous molecules such as carbohydrate polymers and of course also when the modification comprises addition of side chains or side groups to an polypeptide-derived peptide chain.

For the purpose of recombinant gene technology, and of course also for the purpose of nucleic acid immunization, nucleic acid fragments encoding the necessary epitopic regions and analogues are important chemical products. Hence, an important part of the invention pertains to a nucleic acid fragment which encodes an analogue described above of any of the relevant tumour-specific polypeptides, preferably a polypeptide wherein has been introduced a foreign T_H-cell epitope by means of insertion and/or addition, preferably by means of substitution and/or deletion. The nucleic acid fragments of the invention are either DNA or RNA fragments.

The nucleic acid fragments of the invention will normally be inserted in suitable vectors to form cloning or expression vectors carrying the nucleic acid fragments of the invention; such novel vectors are also part of the invention. Details concerning the construction of these vectors of the invention will be discussed in context of transformed cells and microorganisms below. The vectors can, depending on purpose and type of application, be in the form of plasmids, phages, cosmids, mini-chromosomes, or virus, but also naked DNA which is only expressed transiently in certain cells is an important vector. Preferred cloning and expression vectors of the invention are capable of autonomous replication, thereby enabling high copy-numbers for the purposes of high-level expression or high-level replication for subsequent cloning.

The general outline of a vector of the invention comprises the following features in the 5'-3' direction and in operable linkage: a promoter for driving expression of the nucleic acid

fragment of the invention, optionally a nucleic acid sequence encoding a leader peptide enabling secretion of or integration into the membrane of the polypeptide fragment, the nucleic acid fragment of the invention, and a nucleic acid sequence encoding a terminator. When operating with expression vectors in producer strains or cell-lines it is for the purposes of genetic stability of the transformed cell preferred that the vector when introduced into a host cell is integrated in the host cell genome. In contrast, when working with vectors to be used for effecting *in vivo* expression in an animal (i.e. when using the vector in DNA vaccination) it is for security reasons preferred that the vector is not capable of being integrated in the host cell genome; typically, naked DNA or non-integrating viral vectors are used, the choices of which are well-known to the person skilled in the art.

The vectors of the invention are used to transform host cells to produce the analogue of the invention. Such transformed cells, which are also part of the invention, can be cultured cells or cell lines used for propagation of the nucleic acid fragments and vectors of the invention, or used for recombinant production of the analogues of the invention. Alternatively, the transformed cells can be suitable live vaccine strains wherein the nucleic acid fragment (one single or multiple copies) have been inserted so as to effect secretion or integration into the bacterial membrane or cell-wall of the analogue.

Preferred transformed cells of the invention are microorganisms such as bacteria (such as the species Escherichia [e.g. E.coli], Bacillus [e.g. Bacillus subtilis], Salmonella, or Mycobacterium [preferably non-pathogenic, e.g. M. bovis BCG]), yeasts (such as Saccharomyces cerevisiae), and protozoans. Alternatively, the transformed cells are derived from a multicellular organism such as a fungus, an insect

cell, a plant cell, or a mammalian cell. Most preferred are cells derived from a human being, cf. the discussion of cell lines and vectors below.

For the purposes of cloning and/or optimized expression it is preferred that the transformed cell is capable of replicating the nucleic acid fragment of the invention. Cells expressing the nucleic fragment are preferred useful embodiments of the invention; they can be used for small-scale or large-scale preparation of the analogue or, in the case of non-pathogenic bacteria, as vaccine constituents in a live vaccine.

When producing the analogue of the invention by means of transformed cells, it is convenient, although far from essential, that the expression product is either exported out into the culture medium or carried on the surface of the transformed cell.

When an effective producer cell has been identified it is preferred, on the basis thereof, to establish a stable cell line which carries the vector of the invention and which expresses the nucleic acid fragment encoding the analogue. Preferably, this stable cell line secretes or carries the analogue of the invention, thereby facilitating purification thereof.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with the hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., 1977). The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and

thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the prokaryotic microorganism for expression.

Those promoters most commonly used in recombinant DNA construction include the B-lactamase (penicillinase) and lactose promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et al., 1979) and a tryptophan (trp) promoter system (Goeddel et al., 1979; EP-A-0 036 776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebwenlist et al., 1980). Certain genes from prokaryotes may be expressed efficiently in E. coli from their own promoter sequences, precluding the need for addition of another promoter by artificial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used, and here the promoter should be capable of driving expression. *Saccharomyces cerevisiase*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available such as *Pichia pastoris*. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used (Stinchcomb et al., 1979; Kingsman et al., 1979; Tschemper et al., 1980). This plasmid already contains the trpl gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence of the trpl lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzman et al., 1980) or other glycolytic enzymes (Hess et al., 1968; Holland et al., 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, 1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 293 and MDCK cell lines.

Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., 1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the *Hind*III site toward the *Bgl*I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Compositions of the invention

The invention also relates to an immunogenic composition which comprises, as an effective immunogenic agent at least one of the analogues described herein in admixture with a pharmaceutically and immunologically acceptable carrier,

vehicle, diluent, or excipient, and optionally an adjuvant, cf also the discussion of these entities in the description of the method of the invention above.

Furthermore, the invention also relates to a composition composition for inducing production of antibodies against any one of the above discussed tumour antigens, the composition comprising

- a nucleic acid fragment or a vector of the invention, and
- a pharmaceutically and immunologically acceptable diluent and/or vehicle and/or carrier and/or excipient and/or adjuvant.

Formulation and other specifics concerning such compositions are discussed in the relevant section regarding nucleic acid immunisation above.

EXAMPLE 1

Vaccination agains PSM

In the following it will be described how a human autovaccine against PSM can be developed through modification of the molecule by insertion of one or more promiscuous foreign T cell epitopes to reveal a panel of immunogenised PSM molecules.

The constructs will be tested for their ability to induce specific CTL responses against PSM bearing tumour cells. Furthermore, the constructs will be tested for their ability to induce antibodies which are cross-reactive with the native parts of the PSM molecule. Subsequently, in several *in vitro* assays and *in vivo* animal models the efficacy of the different constructs will be evaluated. The induced antibodies will be

tested for their ability to activate complement via the classical pathway and to initiate ADCC via Fc-receptors. Finally, the different molecules will be tested in animal models of human prostate cancer.

Strategy in designing a PSM autovaccine

Briefly, the PSM vaccination plan entails the following experimental tasks

Design and production of a panel of human PSM mutants

- Cloning of the PSM sequences from human and rat/mouse.
- Mutational work to generate immunogenized hPSM molecules.
- Expression of wild type and immunogenized hPSM molecules in *E. coli* and/or *Pichia pastoris*.
- Purification, refolding and characterization of the immunogenized hPSM molecules.

DNA vaccination feasibility study

Selection of hPSM candidates

- Immunizations of mice/rabbits.
- ELISA.
- FACS analysis.
- Tumor cell proliferative assay.
- T cell presentation assays.

Testing of the hPSM mutants *in vivo*

- solid tumor/metastasis model in mice.

Conceptual study: CTL induction by autovaccination

- Construction of immunogenized mouse/rat PSM corresponding to the selected hPSM candidates.
- Testing the antisera raised by mouse/rat PSM mutants in *in vitro* assays: Immunochemical assays, ELISA, FACS analysis,

cellular assays, complement lysis of PSM bearing cells, ADCC assay, CTL activity assay, Tumor cell proliferative assay, T cell presentation assays.

- Testing of the mPSM mutants *in vivo* in a solid tumor/metastasis model in mice.

Nomenclature of the hPSM constructs

PSM is a type II membrane protein of 750 amino acids, cf. SEQ ID NO: 2 which sets forth the wild-type sequence with the exception that Gly substitutes Trp in position 2 due to the introduction of an *Nco*I site and a Kozak sequence in SEQ ID NO: 1, where ggt substitutes tgg in positions 4-6.

In PSM, the extracellular part constitutes the 707 C-terminal amino acids, whereas the cytoplasmic domain is predicted to be 19 amino acids long and the transmembrane part of the protein consists of 24 amino acids (aa 20-43).

All the genetic constructs of human PSM are designated hPSM__.__, where the first __ is the insertion region used for insertion of P2, and the second __ is the insertion region used for P30. If P2 or P30 is not present in the protein, the number 0 (zero) is designated. The full length wild type hPSM is designated hPSM0.0 and the wild type hPSM lacking the cytoplasmic and transmembrane parts is designated hPSM÷0.0. The 13 planned immunogenized hPSM genes which all contain one P2 epitope and one P30 epitope will be named hPSM1.1, hPSM6.1, hPSM8.1, hPSM10.1, hPSM1.6, hPSM1.8, hPSM1.10, hPSM1.2, hPSM1.3, hPSM1.5, hPSM2.1, hPSM3.1, and hPSM5.1, cf. details below.

In hPSM1.1, both the P2 and the P30 epitopes are inserted in tandem in insertion region no. 1 (the membrane spanning region). Theoretically, this mutant, hPSM1.1, can be

considered a very attractive vaccine candidate, because the whole extracellular domain of this molecule is intact.

In order to facilitate the cloning and mutagenesis procedures, much of the molecular construction work is done using either the N-terminal (amino acids 1-436) or the C-terminal (amino acids 437-750) part of the hPSM gene as starting material. These two parts of the hPSM gene are designated hPSMI__.__ and hPSMII__.__, respectively, where the first __ is the insertion region used for insertion of P2, and the second __ is the insertion region used for P30. Again, if P2 or P30 is not present in the protein, the number 0 (zero) is designated, and the wild types are named hPSMI0.0 and hPSMII0.0, respectively. A special variant of hPSMI0.0 without the cytoplasmic part of hPSM is designated hPSMI÷0.0.

Practically, most mutagenesis work is being done using hPSMI0.0 and hPSMII0.0 as starting material.

The expressed hPSM mutant proteins will be designated PROS__.__, where the first __ is the insertion region used for insertion of P2, and the second __ is the insertion region used for P30. If P2 or P30 is not present in the protein, the number 0 (zero) is designated. The wild type hPSM is designated PROS0.0. PROSII0.0 is the hPSM amino acids 437-750 protein product. HIS tagged proteins are called HIS-PROS__.__.

Cloning of the human PSM sequence

The LNCaP cell line which originates from a metastatic lesion of human prostatic adenocarcinoma was purchased from the American Type Culture Collection (ATCC). mRNA was isolated from this cell line and reverse transcribed using a standard kit in order to obtain cDNA encoding the human PSM sequence. Using different sets of hPSM specific primers, PCR products

encoding PSM(437-750) was obtained and further cloned into pUc19 (plasmid number pMR300) and verified by DNA sequencing. This C-terminal part of wild type PSM is designated hPSM partII (hPSMII0.0).

Similarly, the wild type hPSM partI (hPSMI0.0) has been cloned into pUc19 using primers for amplifying partI both with (hPSMI0.0) and without (hPSMI÷0.0) the transmembrane+cytoplasmic domains. The clones were control sequenced and hPSMI0.0 and hPSMII0.0 were fused using ligation at the EcoRI site. The resulting clones of hPSM0.0 (SEQ ID NO: 2) and hPSM÷0.0 have been subcloned into a number of pro- and eucaryotic expression vectors and again sequence verified. For details of the hPSM wild type cloning work.

Cloning of the rat and mouse PSM sequences

The sequences of both mouse and rat cDNAs with very high homology to the human PSM sequences have previously been submitted to the Genbank database. The accession numbers are: mouse PSM: AF026380 (submitted 28 October 1997) and rat PSM: U75973 (submitted 23 October 1996). Knowledge of these sequences have facilitated the cloning of the mouse PSM as well as the rat PSM, and it is currently aimed at to clone the murine PSM sequence.

cDNA libraries of rat hippocampus, rat prostate and mouse brain have been purchased commercially. The stocks have all been titrated and their titers determined. Phage DNA was extracted from culture, and PCR amplifications using primers which are totally conserved between the published human and rat PSM sequences was performed in an attempt to obtain mouse/rat PSM specific probes for use in the selection of m/rPSM expressing clones, but with no positive result.

The three libraries were subsequently plated out and screened for plaques reactive with two probes consisting of the wild type PCR products comprising hPSMI0.0 and hPSMII0.0, respectively, which were radioactively labelled by incorporation of ^{35}S-ATP in the PCR reaction. Several plaques positive for both probes were identified in each of the screened libraries. Double positive plaques from the rat hippocampus and mouse brain libraries were picked, but the second screening of these plaques with radioactive probes came out negative.

600.000 clones from the mouse brain lambda-gt11 cDNA library has been screened using ^{32}P labelled hPSM÷0.0 as probe. Four positive clones were identified and used as templates for PCR reactions with mouse PSM specific primers. Sequencing of such PCR products revealed that one of the clones contains at least 1300 base pairs in the 3´ end of mouse PSM. Since only one positive clone could be identified in this relatively large number of plaques, at this point no further work will be done using this library.

In order to isolate PSM directly from mouse tissue, prostate glands were dissected from male BALB/c mice. Messenger RNA was isolated from this tissue using an oligo-dT coupled magnetic bead based commercial kit and subsequently reverse transcribed. Using this cDNA as template, several PCR products were obtained using different sets of mouse PSM specific primers. In this way, approximately 1300 base pairs in the 3' end of mouse PSM was so far identified. Ongoing and upcoming work includes attempts to identify mouse PSM sequences in the 5' end of the gene using different primer sets as well as cDNA synthesized from mRNA isolated from mouse macrophages and mouse brain.

Recently, two EST (expressed sequence tag) clones containing mouse PSM sequences (from fetal mouse kidney and mouse macrophages, respectively) have been purchased from American Type Culture Collection (ATCC). From the published sequences it is expected that these clones will cover the 1400 3' base pairs of the mouse PSM molecule.

Expression of wild type hPSM in *E. coli*

The C-terminal part (amino acids 437-750) of hPSM, hPSMII0.0, has been cloned into the bacterial expression vector pET28b in order to obtain a product with an N-terminal poly-histidine (HIS) tail which facilitates easy large scale purification and identification with anti-poly-HIS antibodies. The protein product of poly-HIS tagged hPSMII0.0 (protein product designated HIS-PROSII0.0) was expressed in *E. coli*.

The DNA encoding full-length wild type hPSM0.0 (SEQ ID NO: 1) has also been expressed from pET28b in the *E. coli* strain BL21(DE3) where the expression product is located in inclusion bodies. SDS-PAGE analysis of bacterial lysate followed by Western blotting with rabbit anti-HIS-PROSII0.0 showed a product with the expected migration. N-terminal sequencing of five amino acids of this product eluted from an SDS-PAGE gel gave the expected amino acid sequence.

The wild type hPSM constructs hPSM0.0, hPSM÷0.0 (as well as two hPSM mutants, hPSM1.1 and hPSM6.1, see below) have been cloned into different *E. coli* expression vectors in order to enable a more efficient expression and some degree of folding of the recombinant proteins in *E. coli*. The chosen expression systems are:

pMCT6 which generates N-terminally His-tagged versions of the expressed recombinant proteins,

pGH433 which express the recombinant proteins in connection to a 22 amino acid pelB leader sequence which should direct the protein to the periplasmic space of the *E. coli* bacteria, and

pMal-p2 in which recombinant proteins are expressed as C-terminal fusions to maltose binding protein (MBP) containing the natural MBP periplasmic leader sequence. Antibodies against MBP can be used for detection of the fusion proteins and a carbohydrate coupled column can be used for affinity purification of the product.

However, E.coli expression experiments of the wild type hPSM proteins from these vectors only showed a fair expression level from pMCT6. The problems of getting periplasmic expression of the wild type hPSM proteins are still not solved at present.

Expression of wild type hPSM in *Pichia pastoris*

Because of the relatively high molecular weight of the PSM protein and its relatively high degree of glycosylation (app. 16% of the molecular weight) and in order to facilitate purification by elimination of the refolding step, it has been decided to implement alternative technology for eukaryotic expression of the recombinant proteins. Several well-characterized eukaryotic expression systems have been evaluated, and for the initial screening of hPSM mutants, the yeast *Pichia pastoris* has been chosen as alternative to *E. coli* expression.

An expression system based on the yeast *Pichia pastoris* is currently being implemented. The glycosylation pattern of recombinant proteins expressed in this organism is expected to resemble the mammalian glycosylation patterns more than e.g. *Saccharomyces cerevisiae* due to a lesser branched

mannosylation of the recombinant protein. It has recently been shown that mannose receptor-mediated uptake of antigens by dendritic cells results in an approximately 100-fold more efficient presentation to T cells compared to fluid-phase endocytosed uptake. Therefore, mannosylation might play a role for the antigenicity (and especially the ability to induce CTL responses) of the hPSM mutants and other antigens against which a CTL response is desirable.

A strain of *Pichia pastoris* as well as two different expression vectors have been purchased from Invitrogen. The vector pPICZαA carries a methanol inducible promoter upstream of the polycloning site, whereas the pGAPZαA vector express proteins constitutively. Both vectors encode the α-factor secretion signal in order to export the recombinant proteins to the medium. The selection system of these vectors is zeocin resistance. The sequences encoding hPSM0.0, and hPSM÷0.0 (as well as one hPSM mutant, hPSM1.1, cf. below) were subcloned into these vectors.

Four *Pichia pastoris* strains (X-33, SMD1168, GS115, and KM71) differing e.g. in their growth requirements were transformed with each of these (linearized) plasmids using electroporation. The transformation procedure was repeated several times with minor changes in order to obtain a large number of zeocin resistant clones. Currently, the transformation procedure is being optimized and the screening of already obtained zeocin resistant clones is being performed in order to isolate a hPSM secreting clone. Also, the hPSM genetic constructs mentioned above are subcloned into *Pichia pastoris* vectors with another selection system (HIS dependency) as a backup to the zeocin resistance system.

Expression of wild type hPSM in mammalian cells

An expression system using CHO (chinese hamster ovary) cells will also be implemented for the final testing and production of selected molecules.

CHO strains have been obtained from American Type Culture Collection (ATCC) and the hPSM0.0 gene has been subcloned into the mammalian expression vector pcDNA3.1(-). Other selected constructs (hPSM÷0.0, hPSM1.1) are currently being subcloned into pcDNA3.1(+) as well as another mammalian expression vector pZeoSV2 purchased from Invitrogen. A method to monitor transient expression of wild type hPSM from pcDNA3.1(-) in COS-7 and 293 cells is also being set up at the moment in our cell laboratory under biosafety level class II.

Tissue distribution of hPSM

A commercial kit has been purchased in order to determine whether hPSM expression can be detected in various human tissues including prostate, blood and brain. The method is based on a dot blot detection of polyA containing mRNA extracted from 50 different human tissues. Preliminary results do not indicate hyperexpression of hPSM in tissues such as blood or brain, but the test will be repeated.

Design of the hPSM mutants

When designing the mutational work in PSM, some regions of the protein are very important to preserve in the modified constructs, for example potential and identified T cell epitopes, B cell epitopes and disulfide bridge cysteine residues. Therefore, such "forbidden" regions have been identified within the PSM sequence leaving a limited number of "open" regions of 20 amino acids or more available for exchange with the foreign T helper epitopes P2 and/or P30. Per definition, the transmembrane region is also considered an

"open" region since autoantibodies directed against this region are irrelevant and elimination of this sequence is believed to enhance the solubility of the mutated PSM proteins.

According to our expectation that the autovaccine will induce a CTL response, it would be important to identify and preserve potentially subdominant CTL epitopes in the constructs. Two such epitopes are already known from the literature: 1) the peptide comprising PSM amino acids 4-12 (LLHETDSAV) can be presented on the human MHC class I molecule HLA-A2.1 (*Tjoa 1996*), and 2) the PSM(711-719) (ALFDIESKV) also binds HLA-A2.1 (*ref 25*). We have also searched the PSM amino acid sequence in order to identify primary anchor residues of HLA class I binding motifs as described by Rammensee et al. (*Rammensee, 1995*) for the most abundant HLA class I types (HLA-A1, HLA-A2, HLA-A3, HLA-A23, HLA-A24 and HLA-A28), together constituting 80 % of the HLA-A types of the human population. Likewise, potential HLA-B and HLA-C epitopes have been identified and designated as "forbidden" areas.

Because the initial intention was to use C57/black x SJL F1 hybrid mice in case it was decided to use transgenic mice for testing the PSM autovaccine constructs, potential mouse $H\text{-}2^b$ and $H\text{-}2^s$ T helper epitopes has been identified and considered "forbidden" regions (*Rammensee 1995*).

It is also important to preserve known antibody binding regions in the PSM molecule, because they could be important in the induction of specific anti-PSM autoantibodies. Five such regions have already been described: PSM(63-68), PSM(132-137), PSM(482-487) (WO 94/09820), PSM(716-723) and PSM(1-7) (Murphy, 1996). Using the computer based method of Hopp and Woods for prediction of antigenic determinants, five regions are predicted: PSM(63-69), PSM(183-191), PSM(404-414),

PSM(479-486) and PSM(716-723) (Hopp 1983), some of these overlapping the experimentally found B cell epitopes. These regions will also be preserved in the PSM vaccine candidate molecules.

The PSM protein contains 4 cysteine residues (amino acid positions 22, 196, 466 and 597) which will be preserved in the immunogenized constructs because of their potential importance in the formation of disulfide bridges.

Based on the above mentioned "forbidden" and "open" regions in the hPSM protein, 8 regions suitable for insertion of foreign T helper epitopes were identified:

Insertion regions in **hPSMI** (from initiation site to EcoRI site, aa 1-437):

Region 1: aa 16-52 (4 aa preceding TM, TM (24 aa) and 9 aa after TM = 37 aa)

Region 2: aa 87-108 (22 aa)

Region 3: aa 210-230 (21 aa)

Region 4: aa 269-289 (21 aa)

Region 5: aa 298-324 (27 aa)

Insertion regions in **hPSMII** (from EcoRI site to termination site, aa 437-750):

Region 6: aa 442-465 (24 aa)

Region 7: aa 488-514 (27 aa)

Region 8: aa 598-630 (33 aa)

Region 9: aa 643-662 (20 aa)

Region 10: aa 672-699 (28 aa)

The insertion regions as well as the "forbidden" regions are represented graphically in Fig. 4.

13 different immunogenized PSM constructs will be made by substitution of a segment of amino acids from two of the above listed insertion regions with P2 or P30. Each mutant protein will thus contain both P2 and P30. Experimentally, the mutations will be made in clones of hPSMI and hPSMII cDNA respectively, and the two mutated parts will subsequently be combined by ligation (at the EcoRI site). The P2 and P30 epitopes will be inserted into insertion regions 1, 2, 3, 5, 6, 8 and 10 in order to create the 13 mutants.

The sequences of P2 and P30 are:

P2: QYIKANSKFIGITEL (15 aa), encoded by the nucleotide sequence cag tac atc aaa gct aac tcc aaa ttc atc ggt atc acc **gag ctc** (45 nucleotides), where the sequence in boldface is an *Sac*I site.

P30: FNNFTVSFWLRVPKVSASHLE (21 aa), encoded by the nucleotide sequence ttc aac aac ttc acc gt**a agc ttc** tgg ctg cgt gtt ccg aaa gtt agc gCT **AGC** cac ctg gaa (63 nucleotides), where boldface indicates an *Hind*III site, single underlining indicates an *Eco*47III site, capital letters indicates a *Bst*NI site, and double underlining indicates an *Nhe*I site.

Molecular constructions of the hPSM mutants

Mutations to insert P2 and P30 encoding sequences have been performed using both hPSMI0.0 and hPSMII0.0 as starting material.

In order to generate a majority of the hPSM mutants, P2 and P30 were initially inserted in hPSMI0.0 at insertion position 1. The resulting material (hPSMI1.0 and hPSMI0.1, respectively) was subsequently used as starting material for mutagenesis to insert P2 and P30 at positions 2,3 and 5 and for ligation with epitope mutated hPSMII. hPSMI1.0 was constructed using SOE (single overlap extension) PCR and subsequently sequence verified. hPSMI0.1 was constructed using the "Quick Change" technique and subsequently sequence verified.

The P2 epitope was inserted into positions 2, 3 and 5 of hPSMI1.0 using SOE-PCR to create hPSMI1.2, hPSMI1.3 and hPSMI1.5. These constructions were combined with hPSMII0.0 to create hPSM1.2, hPSM1.3 and hPSM1.5.

hPSMI2.1, hPSMI3.1 and hPSMI5.1 were constructed by SOE PCR using hPSMI0.1 as starting material. This material has been assembled with hSPMII0.0 by ligation at the EcoRI site in order to create the full length mutants hPSM2.1, hPSM3.1 and hPSM5.1.

The P2 epitope was inserted at three different positions of hPSMII0.0 in order to create hPSMII6.0, hPSMII8.0 and hPSMII10.0 using the "Quick Change" technique, and these clones were subsequently sequence verified.

Subsequently, hPSMI0.1 was ligated with hPSMII6.0, hPSMII8.0 and hPSMII10.0 to obtain hPSM6.1, hPSM8.1 and hPSM10.1, and the clones were sequence verified.

Insertion of the P30 epitope in hPSMII is presently being done to generate hPSMII0.6, hPSMII0.8 and hPSMII0.10 using SOE PCR.

hPSM1.1 was constructed using two two-step PCR mutations followed by ligation in a *Hind*III site within the epitope sequence. The full length construct is sequence verified.

In addition to the originally contemplated 13 mutants each containing both P2 and P30, four mutants which only contain a single foreign epitope have been constructed and sequence verified: hPSM1.0, hPSM8.0, hPSM10.0 and hPSM0.1.

A schematic overview of all the generated clones and strains of hPSM wild type DNA and mutants is given in the following two tables.

Cloning and mutagenesis of the human PSM divided into two parts, hPSMI and hPSMII

hPSM material	clone pUC19	clone pTrc99A	clone pET28b	clone pGEM-T	strain pET/Top10	strain pUC/DH5α	strain pTrc/DH5α	strain pET/DH5α	strain pET/BL21	strain pGEM-T/Top10
hPSMI-0.0			pMR301					MR501	MR505	
hPSMI 0.0			pMR304					MR506		
hPSMII 0.0	pMR300	pMR302	pMR303			MR500	MR502	MR503	MR504	
hPSMI 1.0										
hPSMI 1.2										
hPSMI 1.3										
hPSMI 1.5										
hPSMI 0.1			pMR313					MR515		
hPSMI 2.1			p319#12		MR326					
hPSMI 3.1			p320#8		MR327					
hPSMI 5.1			p321		MR328					
hPSMI 1.1				p349						MR562
hPSMII 6.0			pMR306#17					MR508		
hPSMII 8.0			pMR315#18					MR518		
hPSMII 10.0			pMR305#39					MR511		
hPSMII 0.6										
hPSMII 0.8										
hPSMII 0.10										

Construction of hPSM mutants by combination of mutated hPSMI and hPSMII parts

	clone pET28b	clone pMCT6	clone pGEM-T	clone pMal-p2	clone pGH433	strain pET/DH5α	strain pET/Top10	strain pET/BL21	strain pMCT6/Top10	strain pGEM-T/Top10	strain pMal-p2/Top10	strain pGH433/Top10
hPSM0.0	pMR310	p342#4		p354	p351	MR512			MR555		MR569	MR566
hPSM+0.0	pMR312	p343#6		p355	p352	MR514			MR560		MR570	MR567
hPSM1.1	p350	p379		p408	p409		MR563	MR564	MR594		MR596	MR595
hPSM6.1	p316#2	p344#1		p356	p353		MR520	MR521	MR561		MR571	MR568
hPSM8.1	p317						MR522	MR523				
hPSM10.1	p318#2						MR524	MR525				
hPSM1.6												
hPSM1.8												
hPSM1.10												
hPSM1.2	p382						MR597	MR600				
hPSM1.3	p383						MR598	MR601				
hPSM1.5	p407						MR599	MR602				
hPSM2.1	p322						MR529	MR530				
hPSM3.1	p323						MR531	MR532				
hPSM5.1	p324						MR533	MR534				
hPSM8.0	p325						MR535	MR536				
hPSM10.0	p327						MR539	MR540				
hPSM0.1	p326						MR537	MR538				
hPSM1.0	p341#16		p348#4				MR554	MR555		MR556		
hPSM+1.1	p444						MR684					
hPSM-6.1	p445						MR685					

Expression of hPSM mutants in *E. coli*

In small-scale experiments, seven hPSM mutants, hPSM1.1, hPSM6.1, hPSM8.1, hPSM10.1, hPSM2.1, hPSM3.1 and hPSM5.1 were expressed from pET28b in the *E. coli* strain BL21(DE3), and IPTG inducible products of the expected size were identified on Coomassie Blue stained SDS-PAGE gels. However, a product of hPSM1.1 was not detectable. The expression levels of these hPSM mutants were very low compared to the product of the wild type construct hPSM÷0.0. At this point, a fair expression level of the hPSM mutants using the pET system in *E. coli* seems impossible, and the use of other *E. coli* expression systems and/or other host organisms is thus necessary.

As mentioned above, hPSM6.1 and hPSM1.1 have been subcloned into different *E. coli* expression vectors in order to generate

- N-terminally His-tagged versions of the expressed recombinant proteins using vector pMCT6,
- versions of the proteins expressed with the pelB leader sequence which directs the protein to the periplasmic space of the *E. coli* bacteria using vector pGH433, and
- versions of the recombinant proteins expressed as a C-terminal fusion protein to maltose binding protein (MBP) using vector pMal-p2.

So far, a sufficient expression level from any of these constructs has not been obtained.

Since hPSM0.0÷cyt is fairly expressed in *E. coli* while a similar expression level of full length hPSM0.0 or the hPSM mutants has not been observed, it is possible that presence of the cytoplasmic part of the hPSM molecule can somehow "inhibit" the expression of the full-length hPSM constructs in *E. coli*. To test this hypothesis, we made two genetic

constructs of hPSM1.1 and hPSM6.1 without cytoplasmic domains. However, in *E.coli* expression experiments there were only weak expression of these ÷cyt gene products.

Expression of hPSM mutants in *Pichia pastoris*

In order to express the hPSM1.1 mutant protein from the yeast *Pichia pastoris*, the hPSM1.1 sequence has been subcloned into the two different expression vectors pPICZαA and pGAPZαA, and the sequences have been verified.

Expression of hPSM mutants in mammalian cells

As mentioned above, hPSM1.1 is currently being subcloned into the mammalian expression vectors pcDNA3.1(+) and pZeoSV2 and these constructs could be used for expression in e.g. CHO cells.

DNA vaccination

DNA vaccination would, if effective, be very well suited for the PSM autovaccine - especially because this administration form has been shown to promote both CTL mediated immune reactions and antibody production. Therefore, it was the intention to perform a parallel study with the aim of investigating the effect of DNA-vaccination of mice with appropriate vectors encoding immunogenized mouse ubiquitin and/or mouse TNFα. DNA vaccination with hPSM (and/or mutants) encoding naked DNA will also be done.

Feasibility study using immunogenized ubiquitin for DNA vaccination

A feasibility study stating the effect of DNA vaccination with an immunogenized self protein was performed using ubiquitin

with an inserted T helper epitope from ovalbumin (UbiOVA) as a model protein. Sequences encoding UbiOVA as well as wild type ubiquitin were subcloned into the mammalian expression vector pcDNA3.1(-).

Groups of 5 BALB/c mice were immunized with 40 µg pcDNA-UbiOVA or pcDNA-ubiquitin construct either intramuscularly in the quadriceps or intradermally. An control control group of received UbiOVA protein in complete Freunds adjuvant. Three and six weeks later, the immunizations were repeated with the only difference that the UbiOVA protein was emulsified in incomplete Freunds adjuvant.

The mice were bled regularly and the anti-ubiquitin antibody titers were determined. In the DNA vaccinated UbiOVA groups, only very weak anti-ubiquitin antibody titers were obtained. Subsequently, all groups were boosted with UbiOVA protein in incomplete Freunds adjuvant and bled in order to determine whether DNA vaccination with UbiOVA (and not ubiquitin) could potentiate the antibody response towards UbiOVA protein. The results of this experiment showed that there was no significant difference between the UbiOVA groups and the control groups, all mice developed strong anti-ubiquitin antibodies upon this single UbiOVA/FIA boost.

DNA vaccination using hPSM constructs

Currently, mammalian expression vectors are constructed for the purpose of performing a DNA vaccination experiment using hPSM constructs. hPSM0.0, hPSM÷0.0 and hPSM1.1 are subcloned into pcDNA3.1(+) and pZeoSV2. We intend also to include leader sequences to allow secretion of the expressed hPSM proteins *in vivo*.

Purification/characterization of HIS-tagged hPSM(437-750) (HIS-PROSII0.0)

HIS-tagged wild type hPSMII (HIS-PROSII0.0) was expressed from pET28b, and solubilized inclusion bodies were applied to a gel filtration FPLC column and eluded in a buffer containing 8 M urea. Fractions predominantly containing hPSMII were subjected to various refolding conditions to optimize the procedure. Solubilized product dialyzed against a Tris buffer was estimated to be more than 90 % pure using silver-stained SDS-PAGE.

Rabbits were immunized with the purified HIS-PROSII0.0 in order to use the resulting antiserum for detection and possibly affinity purification of the hPSM mutants.

Purification/characterization of soluble hPSM (PROS÷0.0)

Wild type hPSM lacking the cytoplasmic and transmembrane parts, PROS÷0.0, has been expressed in the *E. coli* strain BL21(DE3) upon induction with IPTG and could be detected in inclusion bodies. SDS-PAGE of this bacterial lysate followed by Western blotting with mouse anti-HIS-PROSII0.0 showed a product with the expected migration. N-terminal sequencing of the first five amino acids of this product eluted from an SDS-PAGE gel showed the expected sequence corresponding to human PSM. The product was subjected to a large series of solubilization and refolding experiments. A product which stay in solution can be obtained in a Tris buffer without denaturant or reductant, but SDS-PAGE analysis reveals that the material probably forms large aggregates. However, it is still the intention to immunize mice and rabbits with this material in order to get antibody against hPSM e.g. for analytical purposes.

Currently, we are trying to apply a refolding system based on a Ni-chelate column to the purification of a HIS-tagged version of PROS÷0.0 (HIS-PROS÷0.0).

A batch of washed *E. coli* inclusion bodies of PROS÷0.0 has been prepared for immunization of rabbits to generate a polyclonal antiserum against PSM (see section 3.5.2). Approximately 50% of the protein content in the wet pelleted material contained was PROS÷0.0.

Preparation of KLH-conjugated hPSM peptides for immunization

Three 15-mer peptides were synthesized in order to make an immunogenic conjugate of known hPSM B cell epitopes with an immunological carrier molecule to obtain a polyclonal antiserum which is able to recognize hPSM. These peptides contain the PSM B cell epitope plus 5-6 flanking PSM amino acids in each end.

The peptides were made by automatic synthesis, HPLC purified and control-sequenced using Edman degradation.

A chemically linked conjugate was prepared by cross-linking the B cell epitope containing hPSM peptides KLH using a standard 1-step procedure with glutaraldehyde as the cross-linking agent.

Synthesis of P2 and P30 peptides with flanking hPSM sequences

Six peptides have been designed which correspond to the P2 and P30 epitopes with 5 flanking hPSM amino acids in each end. The flanking amino acids correspond to the epitope insertion sites 6, 8 and 10. The peptides will be used in *e.g.* T cell proliferation assays, but also for other purposes such as ELISA or other *in vitro* assays. The peptide sequences are:

PSMpep007 P2 inserted in hPSM insertion position 6
QERGVQYIKANSKFIGITELRVDCT

PSMpep008 P2 inserted in hPSM insertion position 8
AVVLRQYIKANSKFIGITELEMKTY

PSMpep009 P2 inserted in hPSM insertion position 10
MFLERQYIKANSKFIGITELHVIYA

PSMpep010 P30 inserted in hPSM insertion position 6
NSRLLFNNFTVSFWLRVPKVSASHLEVDCTP

PSMpep011 P30 inserted in hPSM insertion position 8
VVLRKFNNFTVSFWLRVPKVSASHLESFDSL

PSMpep012 P30 inserted in hPSM insertion position 10
LMFLEFNNFTVSFWLRVPKVSASHLEPSSHN

The P2 and P30 epitopes are underlined. The peptides were made by automatic synthesis and are currently in the process of HPLC purification followed by control-sequencing using Edman degradation.

Immunogenicity assays

Different experimental setups have been initiated in order to produce materials and establish immunogenicity assays for the future testing of and selection between the mutated PSM constructs.

Generation of polyclonal rabbit anti-HIS-PROSII0.0 and anti-KLH-PSM-peptide conjugate antisera

Two rabbits were immunized with purified HIS-PROSII0.0, the HIS-tagged C-terminal part of the hPSM protein (amino acids 437-750) emulsified 1:1 with complete Freunds adjuvant and boosted twice (at days 28 and 55) with the same antigen emulsified in incomplete Freunds adjuvant.

Two rabbits were immunized with a cocktail consisting of the KLH-PSM peptide conjugate plus each of the three free peptides. These three peptides each contain a previously defined B cell epitope of hPSM. The cocktail was emulsified 1:1 with complete Freunds adjuvant. The rabbits were boosted twice (at days 28 and 55) with the same antigen emulsified in incomplete Freunds adjuvant.

Cross-reactivity between anti-HIS-PROSII0.0 and PSMpep005 and cross-reactivity between anti-KLH-PSM peptide conjugate plus peptides and HIS-PROSII0.0 was demonstrated in ELISA assays. The anti-HIS-PROSII0.0 antibody has the ability to recognize native hPSM in lysates of LNCaP cells in Western blotting.

Immunization of rabbits with *E. coli* inclusion bodies containing PROS÷0.0

Two rabbits have been immunized and boosted several times with washed *E. coli* inclusion bodies of PROS÷0.0. Sera was obtained, but they did not react with hPSM in Western blotting. These rabbits will next time be boosted with with a preparation of soluble, but aggregated PROS÷0.0 (see section 3.4.2) to hopefully enhance the hPSM reactivity of the antisera.

Immunization of mice with retrovirally expressed hPSM0.0

At this stage of the PSM project, a serious obstacle is still the lack of antibodies which are able to recognize correctly

folded native hPSM. Therefore, an immunization experiment using retrovirally expressed hPSM0.0 was performed.

Six groups of Balb/c mice were immunized with either: 1) mitomycin C treated BALB/c fibrosarcoma cells (79.24.H8) transduced with hPSM0.0 cDNA (CMV-Koz-hPSM), 2) mitomycin C treated BALB/c fibrosarcoma cells (79.24.H8), transduced with empty vector (CMVBipep), 3) packaging cells (BOSC) transfected with hPSM0.0 cDNA (CMV-Koz-hPSM), 4) packaging cells (BOSC) transfected with empty vector (CMVBipep), 5) retrovirus stock expressing hPSM0.0 cDNA (CMV-Koz-hPSM) or 6) retrovirus stock, empty vector (CMVBipep).

At several time points, the mice were bled and the sera obtained tested for reactivity in ELISA for reactivity against HIS-PROSII0.0. Unfortunately, none of the mice developed antibodies able to specifically recognize the HIS-PROSII0.0 preparation.

Generation of monoclonal anti-hPSM antibodies

In order to produce a panel of monoclonal antibodies for the use in detection and analysis of mutated and wild type hPSM proteins, immunization of mice has been initiated. BALB/c mice were immunized by subcutaneous injection of 100 µg purified HIS-PROSII0.0, the HIS-tagged C-terminal part of the hPSM protein (amino acids 437-750). The protein was emulsified with an equal volume of complete Freund's adjuvant for the initial immunization, and with incomplete Freund's adjuvant for the two following booster injections (at days 14 and 29). The last booster injection at 3 days before the cell fusion will be performed using $5x10^6$ irradiated LNCaP cells in PBS per mouse injected intraperitoneally.

However, this experiment was discontinued until a well-characterized full-length wild type hPSM is expressed and purified.

Generation of anti-hPSM polyclonal hybridomas

We are currently preparing an experiment where mice will be immunized with a preparation of soluble, but aggregated PROS÷0.0 in a physiological buffer plus FCA. After several boostings, spleen cells of these mice will be fused to e.g. X63 myeloma cells. The intention is to use antibodies secreted from lines of such fused cells directly for analysis purposes without cloning of the hybridoma cells.

Establishment of an anti-hPSM ELISA

Purified HIS-PROSII0.0 was used for coating polystyrene microtitre plates (Maxisorp) for the purpose of establishing an ELISA assay for testing *e.g.* hybridoma supernatants or mouse and rabbit antisera. Sera from BALB/c mice immunized with the same preparation of HIS-PROSII0.0 were reactive with the immobilized HIS-PROSII0.0 at 0.5 µg per well using horse radish peroxidase labelled rabbit anti-mouse Ig as secondary antibody.

As mentioned above, the ability of an antiserum raised in rabbits against KLH-PSMpep004-PSMpep005-PSMpep006 conjugate mixed with the free peptides to react with immobilized HIS-PROSII0.0 was demonstrated using this ELISA assay.

Using AquaBind® microtitre plates (cf. the disclosure in WO 94/03530 describing *i.a.* microtitre surfaces coated with tresyl-activated dextran which are now marketed under the registered trademark AquaBind), an ELISA using immobilized PSM peptides (PSMpep004, PSMpep005 and PSMpep006) was established.

AquaBind® plates coated with these peptides could detect a rabbit antiserum raised against the same preparation of antigen. As mentioned above, rabbit anti-HIS-PROSII0.0 could be detected on AquaBind® plates coated with PSMpep005.

Establishment of an anti-hPSM Western blot using LNCaP cells and monoclonal antibody 7E11C5

7E11C5 B cell hybridomas which secrete mouse IgG2a monoclonal antibody against an intracellular epitope of human PSM was purchased from ATCC. Culture supernatant from approximately 90 % dead cells was collected and used in Western blotting for detection of human PSM in both membrane enriched preparations of LNCaP cells as well as in LNCaP cell lysates. This antibody was purified using protein G columns, and its reactivity with LNCaP in Western blotting verified.

Establishment of a FACS method to detect hPSM on LNCaP cells

We are currently working on establishing a FACS method to detect hPSM on LNCaP cells. Several problems are being addressed: The LNCaP cells grow very slow and in irregular clumps, and therefore the method to prepare single cell suspensions should be optimized. Secondly, the epitope recognized by the mAb 7E11C5 is in the literature defined to be in the cytoplasmic domain of hPSM. Therefore, the method to fix and permeabilize the cells is being developed. For this purpose, protein G purified 7E11C5 antibody has been FITC conjugated and can thus be used without secondary antibody in FACS analysis.

Generation of mouse CTL target cells expressing mTNFα or FGF8b

Since it will probably take some time until mouse PSM mutants are available, it has been decided to perform a parallel study

with the aim of establishing a conceptual proof that an autovaccine constructed by M&E´s patented technology is able to induce a CTL response in the recipient. Immunogenized mouse FGF8b and TNFα molecules will be used as model autovaccines, and CTLs will be isolated from mice immunized with these molecules and tested for their ability to lyse MHC class I positive target cells which express FGF8b/TNFα.

Current work being performed in this study includes the subcloning of murine wild type FGF8b and TNFα cDNA sequences into retroviral vectors. These vectors will be used for intracellular expression of these molecules in target cells which are necessary tools for the detection of FGF8b and TNFα specific CTLs. Murine TNFα cDNA has been cloned into the retroviral vector CMVBipep using BamHI-NcoI adaptors encoding a Kozak sequence. Transductions of CTL target cells P815 ($H\text{-}2^d$) and EL-4 ($H\text{-}2^b$) have been performed using the TNFα vector, and the presence of the retroviral constructs in the transduced cell lines has been verified by PCR analysis on genomic DNA. However, the expressed TNFα gene products has not been detected yet. In parallel, the same target cell lines have been transfected with pcDNA3.1(-) constructs encoding mTNFα cDNA, and geneticin resistant transfectants are currently being cloned.

Establishing a cytotoxicity assay

A method to purify dendritic cells from mouse bone marrow has been implemented. Using model proteins, immunization of mice with dendritic cells pulsed with model class I peptides and protein has been optimized. Also, mice have been immunized with a model protein (β-galactosidase) formulated in the form of ISCOMS. T-cells purified from immunized mice have been *in vitro* restimulated with different forms of the corresponding antigens. The ability of these restimulated CTLs to lyse

different kinds of target cells (including pulsed dendritic cells as well as transfectants expressing retrovirally expressed cytosolic class I peptide) was subsequently measured. Two different *in vitro* assays measuring CTL activity have been established, using either chromium release or and DNA fragmentation (JAM method) as measures of cytotoxicity. Very nice results were obtained with the β-galactosidase model protein and with various combinations of MHC class I and class II binding model peptides.

The conditions for this setup are currently being further optimized, and subsequently a CTL assay using AutoVac™ antigens could be established.

CTL responses raised in mice using AutoVac™ molecules

Preliminary assays have been set up using mouse TNFα AutoVac™ mutants formulated in ICOMS as immunization material, but it was decided to further optimize the procedures with model antigens before proceeding to the use of AutoVac™ molecules for immunization.

***in vivo* PSM tumor models**

Mouse T cell proliferation assays with P2 and P30

A series of T cell proliferation experiments has been initiated in order to establish the T cell immunogenicity of P2 and P30 peptides in various mouse strains (BALB/cA ($H\text{-}2^d$), C3H/Hen ($H\text{-}2^k$), DBA/1 ($H\text{-}2^q$) and C57BL/6 ($H\text{-}2^b$)). It is well known that these epitopes are promiscuous in humans, but the T cell promiscuity also needed to be confirmed in mice using M&Es experimental setup. It was thus shown that P30 is T cell immunogenic in the BALB/cA and C57BL/6 strains whereas neither

P2 or P30 were shown to be T cell immunogenic in the C3H/Hen strain. In DBA/1, T cells could be raised against P2.

Generation of hPSM expressing mouse tumor cells

For the use of a hPSM specific tumor model in mice as well as for the use in tumor cell proliferative assays, a panel of hPSM expressing mouse tumor cells are being established. The intention was to generate these cell lines by transducing the mouse tumor cell lines with retroviral vectors encoding the full-length wild type hPSM0.0 cDNA.

Three different constructs encoding full length wild type cDNA encoding human PSM inserted into the polycloning site of the retroviral vector CMVBipep was constructed, two of these containing a short Kozak sequence upstream of the start codon.

These constructs were transduced into three different mouse tumor cell lines: P815 (mastocytoma, H-2^d), B16-F10 (melanoma, H-2^b) and 79.24.H8 (fibrosarcoma, H-2^d) using the BOSC packaging cell line. Geneticin resistant clones have been obtained for all three cell types, and it was verified in PCR analysis on genomic DNA template that the retroviral constructs were integrated in the host cells. Unfortunately, it has not been possible to detect an expressed PSM gene product in Western blot or FACS analysis using the 7E11C5 monoclonal antibody.

As an alternative to retroviral transduction, traditional stable transfections of the same mouse tumor cell lines was performed using hPSM0.0 cDNA subcloned in the mammalian expression vector pcDNA3.1(÷) under the control of the CMV promoter. Geneticin resistant transfectants were cloned, and currently, a large panel of clones are being tested in Western blot for reactivity with the 7E11C5 monoclonal antibody.

Establishment of a hPSM specific tumor model in mice

It has been decided to establish at least two *in vivo* tumor models in immune competent mice in order to determine the anti-tumor effect of antibodies raised in mice against the immunogenized hPSM molecules. This will hopefully be done by injecting syngeneic mouse tumor cell lines modified to express wild type hPSM on the surface membrane. Cells which form solid tumors and cells which are known to metastasize will be used. Cell lines which can be implanted in syngeneic mice without being rejected due to the presence of the foreign hPSM molecule will be used in the model. The ability of the hPSM vaccines to eliminate such tumor cells will be used for the selection of the hPSM vaccine candidates.

In parallel with the establishment of hPSM expressing cell lines, two different cancer models in mice are currently being established using non-transfected mouse tumor cells. Eventually, the experiences gained from these experiments can be used when performing the ultimate experiments with mouse tumor cell lines transfected to express human PSM.

In one series of experiments, the metastasis model, different doses of B16-F10 ($H\text{-}2^b$) melanoma cells were injected intraveneously in the tail veins of groups of C57Bl/6 ($H\text{-}2^b$) mice. At different time points, 50% of the mice in each group were sacrificed, the lungs were dissected and weighed, and metastases were counted under a dissection microscope. Using these parameters, the metastasis progression was quantified and compared for the two groups, and the result was used for optimizing the number of injected cells to a treatable dose.

In the initial experiments of the solid tumor model, different doses of P815 ($H\text{-}2^d$) mastocytoma cells were injected subcutaneously at the lower right flank of groups of metofane-

anaestesized DBA/2 ($H\text{-}2^d$) mice. The sizes of the established tumors were measured with a caliber measuring two different diameters which were multiplied to give the tumor size in mm^2. These values were compared for the two groups.

Next step will be to optimize the dosage of tumor cells and investigate more tumor models, e.g. the SaIN model in the congenic A/j mouse strain.

Conclusions

In the molecular construction work we have succeeded in cloning of the human PSM gene and cloning of the mouse PSM cDNA is proceeding, although at a slower pace. Fourteen fully sequenced immunogenized hPSM constructs have been constructed. The finished hPSM mutants as well as different wild type hPSM molecules have been expressed in *E. coli*, and it was found and verified that the expression level in *E. coli* is very low. Polyclonal antibodies against the C-terminal half of hPSM have been induced in rabbits. Efforts are being made in order to implement different expression tags (His-tag and maltose binding protein fusion) as well as expression systems alternative to *E. coli* inclusion bodies (*E. coli* periplasmic space, *Pichia pastoris* and CHO cells). The purification, refolding and characterization procedures for the *E. coli* expressed recombinant wild type molecule is ongoing. Useful considerations regarding the DNA vaccine technology have been made, and a preliminary feasibility study was performed. The establishment of several *in vitro* assays which will be used for testing of and selection between the mutated PSM constructs is ongoing, including immunochemical assays and FACS analysis. The establishment of *in vivo* solid tumor and metastasis models in mice using hPSM bearing syngeneic mouse tumor cells has been initiated and cell lines for this purpose are currently being established. An array of T cell

proliferation assays have been performed in order to select the mouse strains for the tumour models. CTL assays are being optimized, and nice results with model antigens have been obtained using different immunization methods and assay conditions. Furthermore, target cells for use in the conceptual study to demonstrate CTL induction by autovaccines are being established.

EXAMPLE 2

Production of a Her-2 autovaccine

A human autovaccine against Her-2 can be developed through modification of the molecule by insertion of one or more promiscuous foreign T cell epitopes to reveal a panel of immunogenised Her-2 molecules. These modified proteins will be tested for their ability to induce antibodies which are cross-reactive with the native parts of the Her-2 molecule. Subsequently, in several *in vitro* assays and *in vivo* animal models, the efficacy of the different constructs (as may be the case with the DNA vaccinnation) and modified proteins will be evaluated. The induction of specific CTL responses against Her-2 bearing tumour cells will be analysed. Also, the induced antibodies will be tested for their ability to activate complement via the classical pathway and to initiate ADCC via Fc-receptors. Finally, the different modified molecules will be tested in animal models of human breast cancer to examine their effects on the treatment of tumours.

Immunogenic rat and human molecules will be constructed with promiscuous T-cell epitopes at different positions in the molecule.

During vaccination against the entire extracellular domain of Her-2 there is a possibility of some degree of cross reaction of the antibodies with other EGFr receptors since some of these receptors are homologous by up to 40-46% in the extracellular domains. Therefore it is planned that the conserved regions of Her-2 would be disrupted by inserting foreign T cell epitopes at least in some of the modified proteins (see below for details).

Regions of Her-2 that may potentially be CTL or B-cell epitopes are avoided in designing of constructs are seen in Fig. 3. The rationale for using these positions is as follows:

The human Her-2 sequence can be divided into a number of domains based solely on the primary structure of the protein.

Extracellular (receptor) part:

1-173: Domain I (N-terminal domain of mature polypeptide).

174-323: Domain II (Cysteine rich domain, 24 cysteine residues).

324-483: Domain III (ligand binding domain i the homologous EGF receptor).

484-623: Domain IV (Cysteine rich domain, 20 cysteine residues).

624-654: Transmembrane domain (TM resudues from 654 - 675).

Intracellular (kinase) part:

655-1010: Tyrosine kinase domain (core TK domain from 725 - 992).

1011-1235: C-terminal domain.

Selection of sites in the amino acid sequence of HER2 to be displaced by either the P2 or P30 human T helper epitopes has been done considering the following parameters (loosely prioritised):

1. Known and predicted CTL epitopes
2. Homology to related receptors (EGFR in particular)
3. Conservation of cysteine residues
4. Predicted loop, α-helix and β-sheet structures
5. Potential N-glycosylation sites
6. Prediction of exposed and buried amino acid residues
7. Domain organisation

The CTL epitopes appear to be clustered in domain I, domain III, the TM domain and in two or three "hot spots" in the TK domain. According to the invention, these should be largely conserved.

Regions with a high degree of homology with other receptors are likely to be structurally important for the "overall" tertiary structure of Her-2, and hence for antibody recognition, whereas regions with low homology possibly can be exchanged with only local alterations of the structure as the consequence.

Cysteine residues are often involved in intramolecular disulphide bridge formation and are thus crucial for the tertiary structure and should preferably not be changed.

Regions predicted to form α-helix or β-sheet structures should preferably be avoided as insertion points of foreign epitopes, as these regions are probably important for the folding of the protein.

Potential N-glycosylation sites should preferably also be conserved because mannosylation of the protein (for example by expression in yeast) is desired, cf. the presence of mannose receptors on APCs.

Regions predicted (by their hydrophobic properties) to be interior in the molecule preferably should be conserved as these could be involved in the folding. In contrast, solvent exposed regions could serve as candidate positions for insertion of the model T_H epitopes P2 and P30.

Finally, the domain organisation of the protein has also been taken into consideration because of its relevance for protein structure and function.

The focus of the strategy has been to conserve the structure of the extracellular part of Her-2 as much as possible, because this is the part of the protein which is relevant as target for neutralising antibodies. By contrast, the intracellular part of native membrane bound Her-2 on the surface of cancer cells is inaccessible for the humoral immune system.

Hence, only the presence of CTL epitopes gives reason to include this part in a vaccine. It is therefore obvious to place one or more epitopes here. If it turns out that it is impossible to express the full length Her-22 molecule in *E. coli* or in yeast, the intracellular part could be truncated after the first CTL epitope "hot spot" (around position 800). Additional CTL epitopes can hereafter be added to the C-terminal end of the truncated molecule.

The transmembrane region probably is an independent folding unit and substitution of this with T_H epitopes such as P2 or P30 will probably not affect the HER2 structure and folding.

In addition, the TM domain might cause great problems for the expression in yeast and coli and should in any case be susbstituted. Thus, an epitope should preferably be placed in this domain in all constructions (perhaps leaving it intact in 1 construction as it contains several CTL epitopes and because it is somehow involved in transmission of signals upon ligand binding).

The extracellular domain could principally be kept intact by placing P2 and P30 in the intracellular and transmembrane domains, thereby maximising the number of potential B-cell epitopes and interfering as little as possible with the structure. However, the high degree of homology to EGFR and Her-3 and Her-4 make a risk for cross reactivity to these receptors which may (or may not) be undesirable. In addition, some monoclonal antibodies have been described which function as agonists for the receptor (perhaps by stimulating heterodimerisation or ligand binding) and increase tumour size, *in vivo*. Positions in the extracellular domain have therefore been selected which thereby hopefully will reduce these risks.

This selection has involved all of the before mentioned parameters and has been based on two different assumptions:
(i) Insertion in non-conserved (with respect to related receptors) regions will help to maintain the tertiary structure and might reduce unwanted activation by antibodies.
(ii) Insertion in the well conserved regions can alter the structure, but might at the same time reduce the risk of cross reactivity by destroying the most related sequences. Both assumptions are speculative, but as it is very difficult to predict the effect of placing an epitope in any position of the protein some of these positions have been included in the constructions.

It has been speculated that it could be an advantage to remove the two cysteine rich domains completely. These are predicted to form solvent exposed loop structures and could form independent folding units perhaps involved in dimerisation (as indicated by the many cysteines that could serve to keep a rigid structure necessary to form a dimerisation domain). Deleting these structures might therefore eliminate the risk of activation by antibodies as well as reduce the number of cross reacting antibodies as these domains are the most well conserved of the extracellular part of the protein. In addition, such cysteine risch domains could be problematic to produce in *E. coli* or yeast cells.

The details of constructs are as follows using the P2 and P30 epitopes as non-limiting examples: the P2 epitope will be placed in the extracellular domain of Her-2 in combination with the P30 epitope substituting part of or all of the membrane spanning region. The P2 epitope will be placed in the 9 region based on the criteria discussed above. The 9 highest and the 5 low priority contstructs are listed below. Also see figure 3 for a diagramatic representation of the construct with highest priorities.

The highest priority Her-2 constructs are:
Her-2 (P2 in positions 61-75), Her-2 (P2 at 105-119), Her-2 (P2 at 151-165), Her-2 (P2 at 212-226), Her-2 (P2 at 252-266), Her-2 (P2 at 327-341), Her-2 (P2 at 371-385), Her-2 (P2 at 467-481), and Her-2 (P2 at 581-595).

The lower priority Her-2 constructs are:
Her-2 (P2 at 72-86), Her-2 (P2 at 146-160), Her-2 (P2 at 221-235), Her-2 (P2 at 257-271), and Her-2 (P2 at 387-401).

Below are described the models that are intended for use in the screening and selection of modified Her-2 proteins.

1. Induction of antibodies in transgenic rat Her-2 mice and in rabbits to rat and human Her-2, respectively, will be investigated by conventional ELISA technology after at least three immunisations. Commercially available antibodies to human and rat Her-2 will be used as positive controls.

2. These rabbit antibodies will be used to study the putative inhibition of growth of human and transgenic mouse tumour cells overexpressing Her-2 in an *in vitro* model.

3. T cell proliferation of PBL from tetanus immunised patients towards selected human Her-2 molecules will be investigated by conventional methods.

4. The ability of modified rat Her-2 molecules to induce CTL responses in rat Her-2 transgenic mice will be studied using tumours from these mice as targets.

5. It is intended to synthesise a selected set of peptides in the transmembrane region of human Her-2 encompassing P2 and P30 epitopes. These peptides will be tested in proliferation of PBL from humans previously immunized with tetenus toxoid to determine whether P2 and P30 epitopes could be efficiently processed out from within the Her-2 sequences and presented to T cells.

6. It is quite possible that selected human modified Her-2 proteins will be tested to generate neutralising antibodies in a mouse that has been genetically constructed to only expresses human VDJ genes. Such a mouse is available from Abgenix, Fremont, CA, U.S.A. as a collaboration.

Four well-characterised transgenic mouse models for breast cancer that contains rat Her-2 oncogene have been described. The first three transgenic mice express activated Her-2 oncogene while the fourth model utilises inactivated Her-2. All models utilise an MMTV promoter to drive expression in mammary glands.

We have decided to use two transgenic mice models: 1) a more aggressive tumour model described by Muller et al using activated Her-2 oncogene (*Muller et al, 1989*) and 2) a less aggressive tumour model in which inactivated Her-2 is used to create focal mammary tumours with long latency (*Guy et al, 1992*). Both transgenic mice are purchased from Jackson and/or Charles Rivers Laboratories.

In the initial experiments, these mice are allowed to produce antibodies and CTL responses by immunising and boosting with modified rat Her-2 proteins. Incidence of tumours will then be investigated as described by others (*Muller et al, 1989; Guy et al, 1992;* Katsumata et al, 1995). Antibody levels will be measured by an ELISA assay. The CTL activity would be determined by generating target cells expressing rat Her-2 as mentioned above.

Alternatively, the nude mouse xenograft carcinoma model can be used for passive vaccination experiments. Nude mice can be transplanted with human tumours and inhibition of tumours could be attempted with passive transfer of serum from normal or humanised mice immunised with modified Her-2 proteins. While this would be useful for studying the role of antibody in suppressing tumours, CTL activity cannot be directly measured in this system.

In the second *in vivo* model, tumours in mice would also be generated by transplanting cells lines from tumours of

transgenic mice described above. Cell lines generated from these mice would be transferred into relevant mouse strain and localisation established using standard protocols.

Transfer of mouse tumours cells over expressing rat Her-2: In this system, cells will be transfected with rat genes and transferred into MHC compatible mice. Inhibition of tumour growth would be achieved by generating anti-Her-2 responses.

In these systems; modified Her-2 proteins would be used as vaccine in adjuvants to generate antibodies and CTL responses.

DNA vaccination has been used successfully in several systems to mount an effective immune response. We are currently investigating means of DNA delivery using modified self proteins. It is our intention to utilise DNA vaccination approach to determine effects of modified Her-2 constructs in inhibiting tumours in transgenic mouse models of breast cancer. Similar approach can than possibly be applied in humans for the treatment of this disease.

EXAMPLE 3

Production of an anti-FGF8b vaccine

In the following it will be described how a human autovaccine against FGF8b can be developed through modification of the molecule by insertion of one or more promiscuous foreign T cell epitopes to reveal a panel of immunogenized "FGF8b" molecules. The constructs will be tested for their ability to induce antibodies that are cross-reactive with the authentic parts of the FGF8b molecule. Subsequently, in several *in vitro* assays and *in vivo* animal models the efficacy of the different constructs will be evaluated. The induced antibodies will be

tested for their ability to activate complement via the classical pathway and to initiate ADCC via Fc-receptors. Finally, the different molecules will be tested in animal models of human prostate and breast cancers.

Construction of an autovaccine against FGF8b

Due to the complete identity of the murine and human FGF8b polypeptides, all constructs can be used for experiments in both humans and mice.

The promiscuous tetanus toxin T helper cell epitopes P2 and P30 used with success in the human TNFα vaccine will be inserted into the FGF8b polypeptide. Due to the small size of FGF8b, constructs will be made with one epitope per molecule. Other promiscuous T helper cell epitopes such as the influenza haemagglutinin epitope HA(307-319) and other T-cell epitopes discussed herein could also be considered (O'Sullivan 1991).

4 different immunogenized FGF8b constructs will be made, with the epitopes distributed along the molecule. These four constructs are made on the basis of the multiple and pairwise alignments shown in Figure 2. The pairwise alignment of FGF2 and FGF8b (Figure 2) is used as basis for an analysis of the presumed secondary structure (i.e. beta-sheet distribution) along the FGF8b molecule. As seen from Figure 4, the residues that are conserved between FGF2 and FGF8b does not cluster anywhere on the three-dimensional structure, which indicates that there are no particular regions of the molecule that cannot be replaced without having deleterious effects on the folding capabilities. The amino acid residues in FGF2 that align to the cysteine residues in FGF8b are positioned very close to each other three-dimensionally (not shown), indicating that they form a disulfide bond in FGF8b. The

flexibility of the N-terminal part of FGF2 was also considered.

The variant of FGF8b with the P30 epitope in the N-terminal (F30N) was designed on the basis of no-gap alignments of the amino acid residues of the FGF8b protein and the P30 epitope (FNNFTVSFWLRVPKVSASHLE), and scoring the different positions with regard to chemical properties of every amino acid residue. Only the region N-terminally of the predicted beta-barrel structure was considered. In the case of F30N, there are 9 similar out of 21 residues. Using this pseudo-algorithm, the substitutions would be expected to result in minimal overall structural changes. The sequences of the four different constructs, as well as three-dimensional representations of the replaced amino acids are shown in Figure 6.

The variant of FGF8b with the P2 epitope (QYIKANSKFIGITEL) in the C-terminal (F2C) was initially designed as F30N. There is, however, predicted a good Kd epitope at positions 195-203. Therefore, the P2 epitope is inserted just C-terminal of this epitope. Again, only the region C-terminal of the predicted beta-barrel was considered.

The internal variants of FGF8b (F30I and F2I) were constructed by replacing external loops in the FGF2 structure with the epitopes P2 and P30, respectively, whereby the beta-barrel structural backbone of the FGF structure presumably will remain unchanged.

The immunogenized FGF8b molecules will be expressed in *Eschericia coli*, which facilitates large scale production of the proteins at minimal costs. Appropriate expression vectors will be obtained commercially. Although, FGF8b contains two potential N-glycosylation sites (Asn31 and Asn177),

bacterially expressed recombinant FGF8b has been shown to be biologically active (MacArthur 1995a, Blunt 1997). In order to facilitate purification and refolding, the FGF8b variants will be produced in a His-tagged version, thereby rendering coupling to a Ni-charged column possible (REFERENCE...)

Purification of the molecules will be performed utilizing the high positive charge of the protein molecules or the His-tag, and refolding will be performed using standard procedures taking the formation of the disulfide bridge into account.

Screening and selection of the modified FGF8b molecules

The four immunogenized FGF8b molecules will be expressed in bacteria and subsequently purified from total cell-lysates or purified inclusion bodies. The different constructs will then be compared for their ability to induce various effects, which are desired in the treatment of prostate and breast cancer patients. Such investigations will be performed using several different *in vitro* and *in vivo* assays. Finally, the results of the experiments will form the basis for the ultimate selection of one or two candidates for a FGF8b vaccine in humans.

***in vitro* models**

Analyses in the murine system

Mice of different haplotypes as well as rabbits will be immunized with the different FGF8b constructs in Complete Freund's Adjuvant and subsequently boosted at least twice with the same antigens emulsified in Incomplete Freunds Adjuvant. Thus, the ability of the different constructs to break B-cell tolerance can be compared.

Serum samples will be obtained at several time points during the immunization schedule, and the ability of the different constructs to induce FGF8b specific antibodies will be determined using a conventional ELISA method (Rochon 1994). A commercial polyclonal antiserum raised against FGF8b (R&D) would be used for positive controls. One monoclonal antibody is described in the literature (Tanaka 1998). Unfortunately, this antibody is not commercially available. The FGF8b protein is commercially available (R&D) but will also be produced along with the other FGF8b constructs and subsequently purified/refolded. This product can then be used for coating of plates in a direct ELISA for testing the sera from mice/rabbits immunized with FGF8b variant proteins.

A valuable tool for investigating the effects of vaccinating against FGF8b will be a FGF8b dependent cancer cell line. Several FGF8b positive cancer cell lines, e.g. MCF-7 or SC-3, are described in the literature. The dependency on FGF8b will be tested in cell proliferation assays using antiserum raised against FGF8b mutants.

The presence of FGF8b ligated to a FGF receptor on the cell surface will be detected with FGF8b specific antibodies in FACS or ELISA analysis. Antibodies directed against several of the different FGF receptors are commercially available (R&D).

The constructs will be compared with respect to their ability to induce antibodies capable of activating complement lysis of FGF8b producing / bearing cells. This can be detected with one of the mouse tumor cell lines expressing FGF8b described earlier. Sera from normal or transgenic mice (see below) immunized with the human FGF8b constructs will be incubated with the cell line and subsequently incubated with fresh guinea pig complement. Antibody mediated complement lysis of the cells can be detected by standard procedures.

The ability of the induced antibodies to mediate ADCC can be studied by measuring the 51Cr-release from labeled FGF8b expressing cells. The effector cells will be PBMC from syngeneic mice. For establishing the assay, it may be convenient to use a mouse cell line capable of mediating ADCC (positive for Fc(-receptors) as effector cell with an antibody against human FGF8b.

In order to show that the FGF8b candidate vaccines do not somehow promote autoantibody induced tumor growth we will also perform a tumor proliferation assay. Serum samples from immunized mice will be incubated with FGF8b expressing tumor cells. Proliferation of the tumor cells can then be detected by their ability to incorporate 3H-thymidine, which subsequently is added to the cells.

Since FGF8b is known to induce proliferation of a range of mammalian cells, it will also be necessary to examine the growth promoting effects of the variant proteins. This can be done using cell proliferation assays as the one used by Marsh 1998.

The biological effect of FGF8b on mammalian cells should be neutralized by the autoantibodies. This can be demonstrated by using recombinant FGF8b and e.g. NIH3T3 in cell proliferation (and morphology changes) studies. Addition of the autoantibodies should abolish the transforming activity of FGF8b.

Immunization protocol

The number of animals that are to enter a FGF8b AutoVac immunization experiment must depend on the expected penetrance of the disease in the model, and thus, the numbers needed to obtain statistically significant information. The immunization

protocol must be based on the experience we have from the TNFa AutoVac project. Various immunization protocols have been used for immunizing mice with the various TNFa analogs for specific purposes, but most experiments were performed using the following protocol:

1. The mice should be individually marked either by earmarks or with transponders, 10 animals in each cage. Presumably, males and females must be evaluated separately, but in any case, we will not have both sexes in the same cage. The animals should be left to rest at least 3 days after transport and marking.

2. Antigen 1 mg/ml in PBS buffer was emulsified with an equal volume Freunds complete antigen (CFA) (Difco or Sigma). The emulsion is checked by placing a drop of the emulsion on a water surface and it is observed whether the drop holds together or disperses. Mixing is maintained until the drop does not disperse.

3. The standard immunization dose is 100µg antigen in a 100µl volume + 100 µl of adjuvant. Thus, the total immunization volume is 200µl, administered s.c. (sub cutaneously) over the back of the animal.

4. Boostings are performed 2-3 weeks after the primary immunization, and subsequently at 2-3 week intervals. The boosting/immunization material is prepared and administered exactly as the immunization material, but Freunds incomplete adjuvant is used. Probably three boosts will induce the maximal titer. Thus, the highest titers will be obtained 6-9 weeks after the first immunization.

5. Bleedings are orbital bleeds of 50-100 µl usually taken before the first immunization and one week after each

boosting. Tail bleeds can also be used, and 10-20 µl can be sufficient for titre determinations.

The initiation point of the immunization program will depend on the development of the disease, and the strategy we want to adopt. Initially, we suggest that it is attempted to generate the maximal immunity as soon as possible, but it is difficult to start immunizations sooner than at approx. 5 weeks of age. Hereafter, high titres should be maintained by boosting at 6-8 week intervals, after the three initial boosts. There is a potential problem if the FGF8b is necessary for the normal development of the young mouse, and therefore one could argue in favor of starting the immunizations later in the adult mouse.

Analyses in the human system

In the selection between the different FGF8b constructs the ability of human antigen presenting cells to present the inserted immunogenic T cell epitopes to human T cells will be investigated. This will be done by using the same *in vitro* processing assays for P2 and P30 presentation that were used for the TNFa vaccine. Human T cell lines, which are specific for P2 and P30, will be established from donors vaccinated against tetanus. Antigen presenting cells (PBMCs) from the same donors will be incubated with the different constructs and T cell lines will be added. The level of presentation of the inserted T cell epitopes can then be compared by measuring the stimulation of the T cell lines.

***in vivo* animal models**

At least two different systems will be used to monitor whether the induced FGF8b antibodies are capable of controlling a FGF8b dependent *in vivo* effect.

The mouse xenograft carcinoma model will be used for passive vaccination experiments. Nude mice will be transplanted with human tumors, and inhibition of tumors would be attempted with transfer of serum from normal or humanized mice immunized with modified FGF8b proteins. This would be very useful for studying the ability of the raised antibodies to suppress tumors.

The other approach to achieve proof of concept will involve the use of mice transgenic for FGF8b. These mice, that are carrying the FGF8b cDNA under control of the very specific mouse mammary tumor virus (MMTV) promoter, are shown to spontaneously develop FGF8b expressing mammary tumors (Coombes personal communication). Autovaccination of these mice with the FGF8b variant proteins would enable us to show if the autovaccine will enable the mice to suppress or reject the tumors.

A third possible approach to obtain proof of concept would be to use the Wnt-1 transgenic mice (MacArthur 1995c). Induction of breast cancers by MMTV virus is reported to activate FGF8 expression in more than half of the mice developing tumors. FGF8b-dependency of the tumors, could be established if our autovaccine(s) could suppress the incidence or growth rate of the tumors.

The fact that transgenic mice often show non-physiological immunological tolerance patterns will most likely not affect this project since the FGF8b polypeptides are identical for human and mouse.

When, and if, a beneficial effect of the induced polyclonal FGF8b antibodies eventually has been demonstrated in the mouse model and suitable human vaccine candidates have been selected it will be possible to perfom a limited number of toxicology

studies. Subsequently, to obtain a final proof of concept, vaccine studies on breast, and prostate cancer patients can be carried out.

Molecular construction work

Four human/murine FGF8b mutants will be constructed.

Mutant construction

A. Construction of the 4 mutant cDNA's (two P2 and two P30) into a bacterial expression vector.

B. Construction of the wild type FGF8b cDNA into a bacterial expression vector

Protein purification

The FGF8b variant proteins will be expressed and purified from E. coli.

Protein purification, refolding, and characterization. This will be continuous work throughout the project starting as soon as the first genetic constructions are made.

in vitro assays

A number of relevant *in vitro* assays will be established for the screening and selection procedure.

ELISA assays

A. Establishment of ELISAs for FGF8b antibodies, both commercial antibodies and autovaccine-generated antibodies. Prove that FGF8b mutant molecules can induce antibody generation.

Cellular assays

A. Establishment of a neutralizing effect of the biological effect of recombinant FGF8b, mediated by the autoantibodies using e.g. NIH3T3 cells.

B. Identification of human breast and prostate cancer cell lines that produce, and are dependent on FGF8b, using antibodies and cell proliferation assays.

C. Inhibition of growth of at least one of these FGF8b dependent cell lines by autovac-cine-generated antibodies, using cell proliferation assays.

D. Development of T cell epitope presentation assays, PBMC T cell assays towards the modified FGF8b antigens and peptides containing the T cell epitopes + flanking regions.

in vivo assays

Suppression or elimination of FGF8b dependent tumors in FGF8 transgenic mice.

Suppression or elimination of FGF8b dependent tumors transplanted into nude mice using autoantibodies.

Critical decision points

Selection of an FGF8b mutant that can induce autoantibodies.

The ability of these autoantibodies to neutralize the biological effect of FGF8b.

Identification of a significant percentage of breast and or prostate tumors that produce and are dependent on FGF8b.

The ability of the autoantibodies to suppress or eliminate FGF8b dependent tumors in FGF8b transgenic mice.

The ability of the autoantibodies to suppress or eliminate FGF8b dependent tumors in nude mice.

If the experiments using *in vivo* models have positive outcome, more mutants will be constructed based on the data available.

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        675                 680                 685

cat gtc atc tat gct cca agc agc cac aac aag tat gca ggg gag tca   2112
His Val Ile Tyr Ala Pro Ser Ser His Asn Lys Tyr Ala Gly Glu Ser
    690                 695                 700

ttc cca gga att tat gat gct ctg ttt gat att gaa agc aaa gtg gac   2160
Phe Pro Gly Ile Tyr Asp Ala Leu Phe Asp Ile Glu Ser Lys Val Asp
705                 710                 715                 720

cct tcc aag gcc tgg gga gaa gtg aag aga cag att tat gtt gca gcc   2208
Pro Ser Lys Ala Trp Gly Glu Val Lys Arg Gln Ile Tyr Val Ala Ala
                725                 730                 735

ttc aca gtg cag gca gct gca gag act ttg agt gaa gta gcc taa       2253
Phe Thr Val Gln Ala Ala Ala Glu Thr Leu Ser Glu Val Ala
            740                 745                 750
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<210> 2
<211> 750
<212> PRT

<213> Homo sapiens

<400> 2

```
Met Gly Asn Leu Leu His Glu Thr Asp Ser Ala Val Ala Thr Ala Arg
  1               5                  10                  15

Arg Pro Arg Trp Leu Cys Ala Gly Ala Leu Val Leu Ala Gly Gly Phe
             20                  25                  30

Phe Leu Leu Gly Phe Leu Phe Gly Trp Phe Ile Lys Ser Ser Asn Glu
         35                  40                  45

Ala Thr Asn Ile Thr Pro Lys His Asn Met Lys Ala Phe Leu Asp Glu
     50                  55                  60

Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr Gln Ile
 65                  70                  75                  80

Pro His Leu Ala Gly Thr Glu Gln Asn Phe Gln Leu Ala Lys Gln Ile
                 85                  90                  95

Gln Ser Gln Trp Lys Glu Phe Gly Leu Asp Ser Val Glu Leu Ala His
            100                 105                 110

Tyr Asp Val Leu Leu Ser Tyr Pro Asn Lys Thr His Pro Asn Tyr Ile
        115                 120                 125

Ser Ile Ile Asn Glu Asp Gly Asn Glu Ile Phe Asn Thr Ser Leu Phe
    130                 135                 140

Glu Pro Pro Pro Pro Gly Tyr Glu Asn Val Ser Asp Ile Val Pro Pro
145                 150                 155                 160

Phe Ser Ala Phe Ser Pro Gln Gly Met Pro Glu Gly Asp Leu Val Tyr
                165                 170                 175

Val Asn Tyr Ala Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg Asp Met
            180                 185                 190

Lys Ile Asn Cys Ser Gly Lys Ile Val Ile Ala Arg Tyr Gly Lys Val
        195                 200                 205

Phe Arg Gly Asn Lys Val Lys Asn Ala Gln Leu Ala Gly Ala Lys Gly
    210                 215                 220

Val Ile Leu Tyr Ser Asp Pro Ala Asp Tyr Phe Ala Pro Gly Val Lys
225                 230                 235                 240

Ser Tyr Pro Asp Gly Trp Asn Leu Pro Gly Gly Gly Val Gln Arg Gly
                245                 250                 255

Asn Ile Leu Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro Gly Tyr
            260                 265                 270
```

```
Pro Ala Asn Glu Tyr Ala Tyr Arg Arg Gly Ile Ala Glu Ala Val Gly
        275                 280                 285

Leu Pro Ser Ile Pro Val His Pro Ile Gly Tyr Tyr Asp Ala Gln Lys
    290                 295                 300

Leu Leu Glu Lys Met Gly Gly Ser Ala Pro Pro Asp Ser Ser Trp Arg
305                 310                 315                 320

Gly Ser Leu Lys Val Pro Tyr Asn Val Gly Pro Gly Phe Thr Gly Asn
                325                 330                 335

Phe Ser Thr Gln Lys Val Lys Met His Ile His Ser Thr Asn Glu Val
            340                 345                 350

Thr Arg Ile Tyr Asn Val Ile Gly Thr Leu Arg Gly Ala Val Glu Pro
        355                 360                 365

Asp Arg Tyr Val Ile Leu Gly Gly His Arg Asp Ser Trp Val Phe Gly
    370                 375                 380

Gly Ile Asp Pro Gln Ser Gly Ala Ala Val Val His Glu Ile Val Arg
385                 390                 395                 400

Ser Phe Gly Thr Leu Lys Lys Glu Gly Trp Arg Pro Arg Arg Thr Ile
                405                 410                 415

Leu Phe Ala Ser Trp Asp Ala Glu Glu Phe Gly Leu Leu Gly Ser Thr
            420                 425                 430

Glu Trp Ala Glu Glu Asn Ser Arg Leu Leu Gln Glu Arg Gly Val Ala
        435                 440                 445

Tyr Ile Asn Ala Asp Ser Ser Ile Glu Gly Asn Tyr Thr Leu Arg Val
    450                 455                 460

Asp Cys Thr Pro Leu Met Tyr Ser Leu Val His Asn Leu Thr Lys Glu
465                 470                 475                 480

Leu Lys Ser Pro Asp Glu Gly Phe Glu Gly Lys Ser Leu Tyr Glu Ser
                485                 490                 495

Trp Thr Lys Lys Ser Pro Ser Pro Glu Phe Ser Gly Met Pro Arg Ile
            500                 505                 510

Ser Lys Leu Gly Ser Gly Asn Asp Phe Glu Val Phe Phe Gln Arg Leu
        515                 520                 525

Gly Ile Ala Ser Gly Arg Ala Arg Tyr Thr Lys Asn Trp Glu Thr Asn
    530                 535                 540

Lys Phe Ser Gly Tyr Pro Leu Tyr His Ser Val Tyr Glu Thr Tyr Glu
545                 550                 555                 560
```

```
Leu Val Glu Lys Phe Tyr Asp Pro Met Phe Lys Tyr His Leu Thr Val
                565                 570                 575

Ala Gln Val Arg Gly Gly Met Val Phe Glu Leu Ala Asn Ser Ile Val
            580                 585                 590

Leu Pro Phe Asp Cys Arg Asp Tyr Ala Val Val Leu Arg Lys Tyr Ala
        595                 600                 605

Asp Lys Ile Tyr Ser Ile Ser Met Lys His Pro Gln Glu Met Lys Thr
    610                 615                 620

Tyr Ser Val Ser Phe Asp Ser Leu Phe Ser Ala Val Lys Asn Phe Thr
625                 630                 635                 640

Glu Ile Ala Ser Lys Phe Ser Glu Arg Leu Gln Asp Phe Asp Lys Ser
                645                 650                 655

Asn Pro Ile Val Leu Arg Met Met Asn Asp Gln Leu Met Phe Leu Glu
            660                 665                 670

Arg Ala Phe Ile Asp Pro Leu Gly Leu Pro Asp Arg Pro Phe Tyr Arg
        675                 680                 685

His Val Ile Tyr Ala Pro Ser Ser His Asn Lys Tyr Ala Gly Glu Ser
    690                 695                 700

Phe Pro Gly Ile Tyr Asp Ala Leu Phe Asp Ile Glu Ser Lys Val Asp
705                 710                 715                 720

Pro Ser Lys Ala Trp Gly Glu Val Lys Arg Gln Ile Tyr Val Ala Ala
                725                 730                 735

Phe Thr Val Gln Ala Ala Ala Glu Thr Leu Ser Glu Val Ala
            740                 745                 750
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<210> 3
<211> 3768
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(3765)

<400> 3

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atg gag ctg gcg gcc ttg tgc cgc tgg ggg ctc ctc ctc gcc ctc ttg   48
Met Glu Leu Ala Ala Leu Cys Arg Trp Gly Leu Leu Leu Ala Leu Leu
  1               5                  10                  15

ccc ccc gga gcc gcg agc acc caa gtg tgc acc ggc aca gac atg aag   96
Pro Pro Gly Ala Ala Ser Thr Gln Val Cys Thr Gly Thr Asp Met Lys
            20                  25                  30
```

```
ctg cgg ctc cct gcc agt ccc gag acc cac ctg gac atg ctc cgc cac   144
Leu Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His
         35                  40                  45

ctc tac cag ggc tgc cag gtg gtg cag gga aac ctg gaa ctc acc tac   192
Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr
     50                  55                  60

ctg ccc acc aat gcc agc ctg tcc ttc ctg cag gat atc cag gag gtg   240
Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val
 65                  70                  75                  80

cag ggc tac gtg ctc atc gct cac aac caa gtg agg cag gtc cca ctg   288
Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu
                 85                  90                  95

cag agg ctg cgg att gtg cga ggc acc cag ctc ttt gag gac aac tat   336
Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
            100                 105                 110

gcc ctg gcc gtg cta gac aat gga gac ccg ctg aac aat acc acc cct   384
Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro
        115                 120                 125

gtc aca ggg gcc tcc cca gga ggc ctg cgg gag ctg cag ctt cga agc   432
Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser
    130                 135                 140

ctc aca gag atc ttg aaa gga ggg gtc ttg atc cag cgg aac ccc cag   480
Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
145                 150                 155                 160

ctc tgc tac cag gac acg att ttg tgg aag gac atc ttc cac aag aac   528
Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
                165                 170                 175

aac cag ctg gct ctc aca ctg ata gac acc aac cgc tct cgg gcc tgc   576
Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys
            180                 185                 190

cac ccc tgt tct ccg atg tgt aag ggc tcc cgc tgc tgg gga gag agt   624
His Pro Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser
        195                 200                 205

tct gag gat tgt cag agc ctg acg cgc act gtc tgt gcc ggt ggc tgt   672
Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys
    210                 215                 220

gcc cgc tgc aag ggg cca ctg ccc act gac tgc tgc cat gag cag tgt   720
Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys
225                 230                 235                 240

gct gcc ggc tgc acg ggc ccc aag cac tct gac tgc ctg gcc tgc ctc   768
Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys Leu
```

```
                  245                 250                 255

cac ttc aac cac agt ggc atc tgt gag ctg cac tgc cca gcc ctg gtc   816
His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu Val
            260                 265                 270

acc tac aac aca gac acg ttt gag tcc atg ccc aat ccc gag ggc cgg   864
Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg
        275                 280                 285

tat aca ttc ggc gcc agc tgt gtg act gcc tgt ccc tac aac tac ctt   912
Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu
    290                 295                 300

tct acg gac gtg gga tcc tgc acc ctc gtc tgc ccc ctg cac aac caa   960
Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln
305                 310                 315                 320

gag gtg aca gca gag gat gga aca cag cgg tgt gag aag tgc agc aag   1008
Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys
                325                 330                 335

ccc tgt gcc cga gtg tgc tat ggt ctg ggc atg gag cac ttg cga gag   1056
Pro Cys Ala Arg Val Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu
            340                 345                 350

gtg agg gca gtt acc agt gcc aat atc cag gag ttt gct ggc tgc aag   1104
Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys
        355                 360                 365

aag atc ttt ggg agc ctg gca ttt ctg ccg gag agc ttt gat ggg gac   1152
Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp
    370                 375                 380

cca gcc tcc aac act gcc ccg ctc cag cca gag cag ctc caa gtg ttt   1200
Pro Ala Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe
385                 390                 395                 400

gag act ctg gaa gag atc aca ggt tac cta tac atc tca gca tgg ccg   1248
Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro
                405                 410                 415

gac agc ctg cct gac ctc agc gtc ttc cag aac ctg caa gta atc cgg   1296
Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg
            420                 425                 430

gga cga att ctg cac aat ggc gcc tac tcg ctg acc ctg caa ggg ctg   1344
Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln Gly Leu
        435                 440                 445

ggc atc agc tgg ctg ggg ctg cgc tca ctg agg gaa ctg ggc agt gga   1392
Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly Ser Gly
    450                 455                 460
```

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ctg gcc ctc atc cac cat aac acc cac ctc tgc ttc gtg cac acg gtg   1440
Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe Val His Thr Val
465                 470                 475                 480

ccc tgg gac cag ctc ttt cgg aac ccg cac caa gct ctg ctc cac act   1488
Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln Ala Leu Leu His Thr
                485                 490                 495

gcc aac cgg cca gag gac gag tgt gtg ggc gag ggc ctg gcc tgc cac   1536
Ala Asn Arg Pro Glu Asp Glu Cys Val Gly Glu Gly Leu Ala Cys His
            500                 505                 510

cag ctg tgc gcc cga ggg cac tgc tgg ggt cca ggg ccc acc cag tgt   1584
Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro Thr Gln Cys
        515                 520                 525

gtc aac tgc agc cag ttc ctt cgg ggc cag gag tgc gtg gag gaa tgc   1632
Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys Val Glu Glu Cys
    530                 535                 540

cga gta ctg cag ggg ctc ccc agg gag tat gtg aat gcc agg cac tgt   1680
Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val Asn Ala Arg His Cys
545                 550                 555                 560

ttg ccg tgc cac cct gag tgt cag ccc cag aat ggc tca gtg acc tgt   1728
Leu Pro Cys His Pro Glu Cys Gln Pro Gln Asn Gly Ser Val Thr Cys
                565                 570                 575

ttt gga ccg gag gct gac cag tgt gtg gcc tgt gcc cac tat aag gac   1776
Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr Lys Asp
            580                 585                 590

cct ccc ttc tgc gtg gcc cgc tgc ccc agc ggt gtg aaa cct gac ctc   1824
Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys Pro Asp Leu
        595                 600                 605

tcc tac atg ccc atc tgg aag ttt cca gat gag gag ggc gca tgc cag   1872
Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu Gly Ala Cys Gln
    610                 615                 620

cct tgc ccc atc aac tgc acc cac tcc tgt gtg gac ctg gat gac aag   1920
Pro Cys Pro Ile Asn Cys Thr His Ser Cys Val Asp Leu Asp Asp Lys
625                 630                 635                 640

ggc tgc ccc gcc gag cag aga gcc agc cct ctg acg tcc atc gtc tct   1968
Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile Val Ser
                645                 650                 655

gcg gtg gtt ggc att ctg ctg gtc gtg gtc ttg ggg gtg gtc ttt ggg   2016
Ala Val Val Gly Ile Leu Leu Val Val Val Leu Gly Val Val Phe Gly
            660                 665                 670

atc ctc atc aag cga cgg cag cag aag atc cgg aag tac acg atg cgg   2064
Ile Leu Ile Lys Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr Met Arg
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```
        675                 680                 685

aga ctg ctg cag gaa acg gag ctg gtg gag ccg ctg aca cct agc gga   2112
Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser Gly
    690                 695                 700

gcg atg ccc aac cag gcg cag atg cgg atc ctg aaa gag acg gag ctg   2160
Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr Glu Leu
705                 710                 715                 720

agg aag gtg aag gtg ctt gga tct ggc gct ttt ggc aca gtc tac aag   2208
Arg Lys Val Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys
                725                 730                 735

ggc atc tgg atc cct gat ggg gag aat gtg aaa att cca gtg gcc atc   2256
Gly Ile Trp Ile Pro Asp Gly Glu Asn Val Lys Ile Pro Val Ala Ile
            740                 745                 750

aaa gtg ttg agg gaa aac aca tcc ccc aaa gcc aac aaa gaa atc tta   2304
Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu
        755                 760                 765

gac gaa gca tac gtg atg gct ggt gtg ggc tcc cca tat gtc tcc cgc   2352
Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg
    770                 775                 780

ctt ctg ggc atc tgc ctg aca tcc acg gtg cag ctg gtg aca cag ctt   2400
Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu
785                 790                 795                 800

atg ccc tat ggc tgc ctc tta gac cat gtc cgg gaa aac cgc gga cgc   2448
Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg
                805                 810                 815

ctg ggc tcc cag gac ctg ctg aac tgg tgt atg cag att gcc aag ggg   2496
Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly
            820                 825                 830

atg agc tac ctg gag gat gtg cgg ctc gta cac agg gac ttg gcc gct   2544
Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu Ala Ala
        835                 840                 845

cgg aac gtg ctg gtc aag agt ccc aac cat gtc aaa att aca gac ttc   2592
Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe
    850                 855                 860

ggg ctg gct cgg ctg ctg gac att gac gag aca gag tac cat gca gat   2640
Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp
865                 870                 875                 880

ggg ggc aag gtg ccc atc aag tgg atg gcg ctg gag tcc att ctc cgc   2688
Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu Arg
                885                 890                 895
```

```
cgg cgg ttc acc cac cag agt gat gtg tgg agt tat ggt gtg act gtg   2736
Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val
            900                 905                 910

tgg gag ctg atg act ttt ggg gcc aaa cct tac gat ggg atc cca gcc   2784
Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro Ala
        915                 920                 925

cgg gag atc cct gac ctg ctg gaa aag ggg gag cgg ctg ccc cag ccc   2832
Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro
    930                 935                 940

ccc atc tgc acc att gat gtc tac atg atc atg gtc aaa tgt tgg atg   2880
Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met
945                 950                 955                 960

att gac tct gaa tgt cgg cca aga ttc cgg gag ttg gtg tct gaa ttc   2928
Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser Glu Phe
                965                 970                 975

tcc cgc atg gcc agg gac ccc cag cgc ttt gtg gtc atc cag aat gag   2976
Ser Arg Met Ala Arg Asp Pro Gln Arg Phe Val Val Ile Gln Asn Glu
            980                 985                 990

gac ttg ggc cca gcc agt ccc ttg gac agc acc ttc tac cgc tca ctg   3024
Asp Leu Gly Pro Ala Ser Pro Leu Asp Ser Thr Phe Tyr Arg Ser Leu
        995                1000                1005

ctg gag gac gat gac atg ggg gac ctg gtg gat gct gag gag tat ctg   3072
Leu Glu Asp Asp Asp Met Gly Asp Leu Val Asp Ala Glu Glu Tyr Leu
   1010                1015                1020

gta ccc cag cag ggc ttc ttc tgt cca gac cct gcc ccg ggc gct ggg   3120
Val Pro Gln Gln Gly Phe Phe Cys Pro Asp Pro Ala Pro Gly Ala Gly
1025                1030                1035                1040

ggc atg gtc cac cac agg cac cgc agc tca tct acc agg agt ggc ggt   3168
Gly Met Val His His Arg His Arg Ser Ser Ser Thr Arg Ser Gly Gly
                1045                1050                1055

ggg gac ctg aca cta ggg ctg gag ccc tct gaa gag gag gcc ccc agg   3216
Gly Asp Leu Thr Leu Gly Leu Glu Pro Ser Glu Glu Glu Ala Pro Arg
            1060                1065                1070

tct cca ctg gca ccc tcc gaa ggg gct ggc tcc gat gta ttt gat ggt   3264
Ser Pro Leu Ala Pro Ser Glu Gly Ala Gly Ser Asp Val Phe Asp Gly
        1075                1080                1085

gac ctg gga atg ggg gca gcc aag ggg ctg caa agc ctc ccc aca cat   3312
Asp Leu Gly Met Gly Ala Ala Lys Gly Leu Gln Ser Leu Pro Thr His
   1090                1095                1100

gac ccc agc cct cta cag cgg tac agt gag gac ccc aca gta ccc ctg   3360
Asp Pro Ser Pro Leu Gln Arg Tyr Ser Glu Asp Pro Thr Val Pro Leu
```

```
1105                1110                1115                1120

ccc tct gag act gat ggc tac gtt gcc ccc ctg acc tgc agc ccc cag   3408
Pro Ser Glu Thr Asp Gly Tyr Val Ala Pro Leu Thr Cys Ser Pro Gln
                1125                1130                1135

cct gaa tat gtg aac cag cca gat gtt cgg ccc cag ccc cct tcg ccc   3456
Pro Glu Tyr Val Asn Gln Pro Asp Val Arg Pro Gln Pro Pro Ser Pro
            1140                1145                1150

cga gag ggc cct ctg cct gct gcc cga cct gct ggt gcc act ctg gaa   3504
Arg Glu Gly Pro Leu Pro Ala Ala Arg Pro Ala Gly Ala Thr Leu Glu
        1155                1160                1165

agg gcc aag act ctc tcc cca ggg aag aat ggg gtc gtc aaa gac gtt   3552
Arg Ala Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp Val
    1170                1175                1180

ttt gcc ttt ggg ggt gcc gtg gag aac ccc gag tac ttg aca ccc cag   3600
Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr Pro Gln
1185                1190                1195                1200

gga gga gct gcc cct cag ccc cac cct cct cct gcc ttc agc cca gcc   3648
Gly Gly Ala Ala Pro Gln Pro His Pro Pro Pro Ala Phe Ser Pro Ala
                1205                1210                1215

ttc gac aac ctc tat tac tgg gac cag gac cca cca gag cgg ggg gct   3696
Phe Asp Asn Leu Tyr Tyr Trp Asp Gln Asp Pro Pro Glu Arg Gly Ala
            1220                1225                1230

cca ccc agc acc ttc aaa ggg aca cct acg gca gag aac cca gag tac   3744
Pro Pro Ser Thr Phe Lys Gly Thr Pro Thr Ala Glu Asn Pro Glu Tyr
        1235                1240                1245

ctg ggt ctg gac gtg cca gtg tga                                  3768
Leu Gly Leu Asp Val Pro Val
    1250                1255
```

<210> 4
<211> 1255
<212> PRT
<213> Homo sapiens

<400> 4

```
Met Glu Leu Ala Ala Leu Cys Arg Trp Gly Leu Leu Leu Ala Leu Leu
  1               5                  10                  15

Pro Pro Gly Ala Ala Ser Thr Gln Val Cys Thr Gly Thr Asp Met Lys
            20                  25                  30

Leu Arg Leu Pro Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His
        35                  40                  45
```

```
Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr
     50                  55                  60

Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val
 65                  70                  75                  80

Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu
                 85                  90                  95

Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
            100                 105                 110

Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro
        115                 120                 125

Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser
    130                 135                 140

Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
145                 150                 155                 160

Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
                165                 170                 175

Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys
            180                 185                 190

His Pro Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser
        195                 200                 205

Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys
    210                 215                 220

Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys
225                 230                 235                 240

Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys Leu
                245                 250                 255

His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu Val
            260                 265                 270

Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg
        275                 280                 285

Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu
    290                 295                 300

Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln
305                 310                 315                 320

Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys
                325                 330                 335
```

Pro Cys Ala Arg Val Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu
340 345 350

Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys
355 360 365

Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp
370 375 380

Pro Ala Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe
385 390 395 400

Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro
405 410 415

Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg
420 425 430

Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln Gly Leu
435 440 445

Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu Gly Ser Gly
450 455 460

Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe Val His Thr Val
465 470 475 480

Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln Ala Leu Leu His Thr
485 490 495

Ala Asn Arg Pro Glu Asp Glu Cys Val Gly Glu Gly Leu Ala Cys His
500 505 510

Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro Thr Gln Cys
515 520 525

Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys Val Glu Glu Cys
530 535 540

Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val Asn Ala Arg His Cys
545 550 555 560

Leu Pro Cys His Pro Glu Cys Gln Pro Gln Asn Gly Ser Val Thr Cys
565 570 575

Phe Gly Pro Glu Ala Asp Gln Cys Val Ala Cys Ala His Tyr Lys Asp
580 585 590

Pro Pro Phe Cys Val Ala Arg Cys Pro Ser Gly Val Lys Pro Asp Leu
595 600 605

Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu Gly Ala Cys Gln
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Ile Leu Ile Lys Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr Met Arg
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Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser Gly
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Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr Glu Leu
705                 710                 715                 720

Arg Lys Val Lys Val Leu Gly Ser Gly Ala Phe Gly Thr Val Tyr Lys
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Gly Ile Trp Ile Pro Asp Gly Glu Asn Val Lys Ile Pro Val Ala Ile
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Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu
        755                 760                 765

Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg
    770                 775                 780

Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu
785                 790                 795                 800

Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg
                805                 810                 815

Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly
            820                 825                 830

Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu Ala Ala
        835                 840                 845

Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe
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Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp
865                 870                 875                 880

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Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val
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930 935 940

Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met
945 950 955 960

Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser Glu Phe
965 970 975

Ser Arg Met Ala Arg Asp Pro Gln Arg Phe Val Val Ile Gln Asn Glu
980 985 990

Asp Leu Gly Pro Ala Ser Pro Leu Asp Ser Thr Phe Tyr Arg Ser Leu
995 1000 1005

Leu Glu Asp Asp Asp Met Gly Asp Leu Val Asp Ala Glu Glu Tyr Leu
1010 1015 1020

Val Pro Gln Gln Gly Phe Phe Cys Pro Asp Pro Ala Pro Gly Ala Gly
1025 1030 1035 1040

Gly Met Val His His Arg His Arg Ser Ser Ser Thr Arg Ser Gly Gly
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Gly Asp Leu Thr Leu Gly Leu Glu Pro Ser Glu Glu Glu Ala Pro Arg
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1075 1080 1085

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Asp Pro Ser Pro Leu Gln Arg Tyr Ser Glu Asp Pro Thr Val Pro Leu
1105 1110 1115 1120

Pro Ser Glu Thr Asp Gly Tyr Val Ala Pro Leu Thr Cys Ser Pro Gln
1125 1130 1135

Pro Glu Tyr Val Asn Gln Pro Asp Val Arg Pro Gln Pro Pro Ser Pro
1140 1145 1150

Arg Glu Gly Pro Leu Pro Ala Ala Arg Pro Ala Gly Ala Thr Leu Glu
1155 1160 1165

Arg Ala Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp Val
1170 1175 1180

Phe Ala Phe Gly Gly Ala Val Glu Asn Pro Glu Tyr Leu Thr Pro Gln
1185 1190 1195 1200

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gtt ctc tgc ctc caa gcc cag gta act gtt cag tcc tca cct aat ttt   96
Val Leu Cys Leu Gln Ala Gln Val Thr Val Gln Ser Ser Pro Asn Phe
             20                  25                  30

aca cag cat gtg agg gag cag agc ctg gtg acg gat cag ctc agc cgc   144
Thr Gln His Val Arg Glu Gln Ser Leu Val Thr Asp Gln Leu Ser Arg
         35                  40                  45

cgc ctc atc cgg acc tac cag ctc tac agc cgc acc agc ggg aag cac   192
Arg Leu Ile Arg Thr Tyr Gln Leu Tyr Ser Arg Thr Ser Gly Lys His
     50                  55                  60

gtg cag gtc ctg gcc aac aag cgc atc aac gcc atg gca gaa gac gga   240
Val Gln Val Leu Ala Asn Lys Arg Ile Asn Ala Met Ala Glu Asp Gly
 65                  70                  75                  80

gac ccc ttc gcg aag ctc att gtg gag acc gat act ttt gga agc aga   288
Asp Pro Phe Ala Lys Leu Ile Val Glu Thr Asp Thr Phe Gly Ser Arg
                 85                  90                  95

gtc cga gtt cgc ggc gca gag aca ggt ctc tac atc tgc atg aac aag   336
Val Arg Val Arg Gly Ala Glu Thr Gly Leu Tyr Ile Cys Met Asn Lys
            100                 105                 110

aag ggg aag cta att gcc aag agc aac ggc aaa ggc aag gac tgc gta   384
Lys Gly Lys Leu Ile Ala Lys Ser Asn Gly Lys Gly Lys Asp Cys Val
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ttc aca gag atc gtg ctg gag aac aac tac acg gcg ctg cag aac gcc   432
Phe Thr Glu Ile Val Leu Glu Asn Asn Tyr Thr Ala Leu Gln Asn Ala
    130                 135                 140

aag tac gag ggc tgg tac atg gcc ttt acc cgc aag ggc cgg ccc cgc   480
Lys Tyr Glu Gly Trp Tyr Met Ala Phe Thr Arg Lys Gly Arg Pro Arg
145                 150                 155                 160

aag ggc tcc aag acg cgc cag cat cag cgc gag gtg cac ttc atg aag   528
Lys Gly Ser Lys Thr Arg Gln His Gln Arg Glu Val His Phe Met Lys
                165                 170                 175

cgc ctg ccg cgg ggc cac cac acc acc gag cag agc ctg cgc ttc gag   576
Arg Leu Pro Arg Gly His His Thr Thr Glu Gln Ser Leu Arg Phe Glu
            180                 185                 190

ttc ctc aac tac ccg ccc ttc acg cgc agc ctg cgc ggc agc cag agg   624
Phe Leu Asn Tyr Pro Pro Phe Thr Arg Ser Leu Arg Gly Ser Gln Arg
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            20                  25                  30

Thr Gln His Val Arg Glu Gln Ser Leu Val Thr Asp Gln Leu Ser Arg
        35                  40                  45

Arg Leu Ile Arg Thr Tyr Gln Leu Tyr Ser Arg Thr Ser Gly Lys His
    50                  55                  60

Val Gln Val Leu Ala Asn Lys Arg Ile Asn Ala Met Ala Glu Asp Gly
65                  70                  75                  80

Asp Pro Phe Ala Lys Leu Ile Val Glu Thr Asp Thr Phe Gly Ser Arg
                85                  90                  95

Val Arg Val Arg Gly Ala Glu Thr Gly Leu Tyr Ile Cys Met Asn Lys
            100                 105                 110

Lys Gly Lys Leu Ile Ala Lys Ser Asn Gly Lys Gly Lys Asp Cys Val
        115                 120                 125
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Phe Thr Glu Ile Val Leu Glu Asn Asn Tyr Thr Ala Leu Gln Asn Ala
    130                 135                 140

Lys Tyr Glu Gly Trp Tyr Met Ala Phe Thr Arg Lys Gly Arg Pro Arg
145                 150                 155                 160

Lys Gly Ser Lys Thr Arg Gln His Gln Arg Glu Val His Phe Met Lys
                165                 170                 175

Arg Leu Pro Arg Gly His His Thr Thr Glu Gln Ser Leu Arg Phe Glu
            180                 185                 190

Phe Leu Asn Tyr Pro Pro Phe Thr Arg Ser Leu Arg Gly Ser Gln Arg
        195                 200                 205

Thr Trp Ala Pro Glu Pro Arg
    210                 215
```

CLAIMS

1. A method for inducing an immune response against a polypeptide antigen in an animal, including a human being, said polypeptide antigen being weakly immunogenic or non-immunogenic in the animal, the method comprising effecting simultaneous presentation by antigen presenting cells (APCs) of the animal's immune system of an immunogenically effective amount of

1) at least one CTL epitope derived from the polypeptide antigen and/or at least one B-cell epitope derived from the cell-associated polypeptide antigen, and

2) at least one first T helper cell epitope (T_H epitope) which is foreign to the animal.

2. A method for down-regulating a cell-associated polypeptide antigen in an animal, including a human being, said polypeptide antigen being weakly immunogenic or non-immunogenic in the animal, by inducing a specific cytotoxic T-lymphocyte (CTL) response against cells carrying the cell-associated polypeptide antigen on their surface or harbouring the cell-associated polypeptide antigen in their intracellular compartment, the method comprising effecting, in the animal, simultaneous presentation by a suitable antigen presenting cell (APC) of

1) at least one CTL epitope derived from the cell-associated polypeptide antigen, and

2) at least one first T-helper lymphocyte (T_H) epitope which is foreign to the animal.

3. The method according to claim 1 or 2, wherein said at least one CTL epitope when presented is associated with an MHC Class I molecule on the surface of the APC.

4. The method according to any of the preceding claims, wherein said at least one first foreign T_H epitope when presented is

associated with an MHC Class II molecule on the surface of the APC.

5. The method according to any of the preceding claims, wherein the APC is a dendritic cell or a macrophage.

6. The method according to any of the preceding claims, wherein the cell-associated antigen is selected from a tumour-associated antigen, a viral antigen, and an antigen derived from an intracellular parasite or bacterium.

7. The method according to any of the preceding claims, wherein presentation by the APC of the CTL epitope and the first foreign T_H epitope is effected by presenting the animal's immune system with at least one first analogue of the cell-associated polypeptide antigen, said first analogue comprising a variation of the amino acid sequence of the cell-associated polypeptide antigen, said variation containing at least the CTL epitope and the first foreign T_H epitope.

8. The method according to claim 7, wherein the at least first analogue contains a substantial fraction of known and predicted CTL epitopes of the cell-associated polypeptide antigen.

9. The method according to claim 8, wherein the substantial fraction of known and predicted CTL epitopes in the amino acid sequence of the analogue are recognized by at least 90% of the MHC-I haplotypes recognizing all known and predicted CTL epitopes in the cell-associated polypeptide antigen.

10. The method according to any of claims 7-9, wherein substantially all known CTL epitopes of the cell-associated polypeptide antigen are present in the analogue.

11. The method according to any of claims 7-10, wherein substantially all predicted CTL epitopes of the cell-associated polypeptide antigen are present in the at least first analogue.

12. The method according to any of claims 7-11, wherein the at least one first analogue further comprises a part consisting of a modification of the structure of the cell-associated polypeptide antigen, said modification having as a result that immunization of the animal with the first analogue induces production of antibodies in the animal against the cell-associated polypeptide antigen.

13. The method according to any one of the preceding claims, which comprises effecting presentation to the animal's immune system of an immunogenically effective amount of at least one second analogue of the cell-associated polypeptide antigen, said second analogue containing a modification of the structure of the cell-associated polypeptide antigen, said modification having as a result that immunization of the animal with the second analogue induces production of antibodies against the cell-associated polypeptide antigen.

14. The method according to claim 13, wherein the modification comprises that at least one second foreign T_H epitope is included in the second analogue.

15. The method according to any one of claims 7-14, wherein the first and/or second analogue(s) comprise(s) a substantial fraction of the cell-associated polypeptide antigen's B-cell epitopes.

16. The method according to any one of claims 7-15, wherein the variation and/or modification involves amino acid substitution and/or deletion and/or insertion and/or addition.

17. The method according to claim 16, wherein the variation and/or modification involves amino acid substitution and/or insertion.

18. The method according to any one of claims 7-17, wherein the variation and/or modification comprises that

- at least one first moiety is included in the first and/or second analogue(s), said first moiety effecting targeting of the analogue to an antigen presenting cell (APC), and/or
- at least one second moiety is included in the first and/or second analogue(s), said second moiety stimulating the immune system, and/or
- at least one third moiety is included in the first and/or second analogue(s), said third moiety optimizing presentation of the analogue to the immune system.

19. The method according to any one of claims 7-18, wherein the variation and/or modification includes duplication of at least one B-cell epitope or of at least one CTL epitope of the cell-associated polypeptide antigen

20. The method according to any one of claims 7-19, wherein the variation and/or modification includes introduction of a hapten.

21. The method according to any of the preceding claims, wherein the first and/or second foreign T_H epitope is/are immunodominant.

22. The method according to any of the preceding claims, wherein the first and/or second foreign T_H epitope(s) is/are promiscuous.

23. The method according to any of claims 14-22, wherein the first and/or second foreign T_H epitope(s) is/are selected from a

natural T_H epitope and an artificial MHC-II binding peptide sequence.

24. The method according to claim 23, wherein the natural T_H epitope is selected from a Tetanus toxoid epitope such as P2 or P30, a diphtheria toxoid epitope, a influenza virus hemagluttinin epitope, and a *P. falciparum* CS epitope.

25. The method according to any one of claims 14-24, wherein the first and/or second T_H epitopes and/or first and/or second and/or third moieties are present in the form of

- side groups attached covalently or non-covalently to suitable chemical groups in the amino acid sequence of the cell-associated polypeptide antigen or a subsequence thereof, and/or
- fusion partners to the amino acid sequence derived from the cell-associated polypeptide antigen.

26. The method according to claim 25, wherein the first moiety is a substantially specific binding partner for an APC specific surface antigen.

27. The method according to claim 26, wherein the first moiety is a carbohydrate for which there is a receptor on the APC, such as mannan or mannose.

28. The method according to any one of claims 18-27, wherein the second moiety is selected from interferon γ (IFN-γ), Flt3L, interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin 13 (IL-13), interleukin 15 (IL-15), and granulocyte-macrophage colony stimulating factor (GM-CSF), or an effective part thereof.

29. The method according to any one of claims 18-28, wherein the third moiety is a lipid such as a palmitoyl residue or a myristyl residue.

30. The method according to claim any one of claims 7-29, wherein the first and/or second analogue(s) has/have substantially the overall tertiary structure of the cell-associated polypeptide antigen.

31. The method according to any one of claims 7-30, wherein presentation by the APC is effected by administering, to the animal, an immunogenically effective amount of the at least one first analogue.

32. The method according to claim 31, wherein is also administered an immunologically effective amount of the at least one second analogue.

33. The method according to claim 31 or 32, wherein said at least one first and/or second analogue(s) is/are formulated together with a pharmaceutically and immunologically acceptable carrier and/or vehicle and/or diluent and/or excipient and, optionally an adjuvant.

34. The method according to claim 30, wherein said adjuvant facilitates uptake by APCs, such as dendritic cells, of the at least first and/or second analogues.

35. The method according to claim 31, wherein the adjuvant is selected from the group consisting of an immune targeting adjuvant; an immune modulating adjuvant such as a toxin, a cytokine, and a mycobacterial derivative; an oil formulation; a polymer; a micelle forming adjuvant; a saponin; an immunostimulating complex matrix (ISCOM matrix); a particle;

DDA; aluminium adjuvants; DNA adjuvants; γ-inulin; and an encapsulating adjuvant.

36. The method according to claim 35, wherein the cytokine is selected from interferon γ (IFN-γ), Flt3L, interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin 13 (IL-13), interleukin 15 (IL-15), and granulocyte-macrophage colony stimulating factor (GM-CSF), or an effective part thereof.

37. The method according to claim 35 or 36, wherein the toxin is selected from the group consisting of listeriolycin (LLO), Lipid A (MPL, L180.5/RalLPS), and heat-labile enterotoxin.

38. The method according to any one of claims 35-37, wherein the mycobacterial derivative is selected from the group consisting of muramyl dipeptide, complete Freund's adjuvant, RIBI, and a diester of trehalose such as TDM and TDE.

39. The method according to any one of claims 35-38, wherein the immune targeting adjuvant is selected from the group consisting of CD40 ligand, CD40 antibodies or specifically binding fragments thereof, mannose, a Fab fragment, and CTLA-4.

40. The method according to any one of claims 35-39, wherein the oil formulation comprises squalene or incomplete Freund's adjuvant.

41. The method according to any one of claims 35-40, wherein the polymer is selected from the group consisting of a carbohydrate such as dextran, PEG, starch, mannan, and mannose; a plastic polymer such as; and latex such as latex beads.

42. The method according to any one of claims 35-41, wherein the saponin is *Quillaja saponaria* saponin, Quil A, and QS21.

43. The method according to any one of claims 35-42, wherein the particle comprises latex or dextran.

44. The method according to any one of claims 31-43, which includes administration via a route selected from the oral route and the parenteral route such as the intradermal, the subdermal, the intracutaneous, the subcutaneous; the peritoneal, the buccal, the sublinqual, the epidural, the spinal, the anal, and the intracranial routes.

45. The method according to any of claim 31-44, which includes at least one administration a year, such as at least 2, 3, 4, 5, 6, and 12 administrations a year.

46. The method according to any of claims 1-6, wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying a nucleic acid fragment encoding and expressing the at least one CTL epitope and the at least one T_H epitope.

47. The method according to any of claims 7-17, wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying at least one nucleic acid fragment which encodes and expresses the at least first analogue.

48. The method according to any of claims 18-30, wherein the T_H epitope and/or the first and/or second and/or third moieties are present in the form of fusion partners to the amino acid sequence derived from the cell-associated polypeptide antigen, and wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying at least one nucleic acid fragment encoding and expressing the first analogue.

49. The method according to any of claims 13-17 or 47, wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying at least one nucleic acid fragment which encodes and expresses the at least first analogue.

50. The method according to any of claims 18-30 or 48, wherein the T_H epitope and/or the first and/or second and/or third moieties are present in the form of fusion partners to the amino acid sequence derived from the cell-associated polypeptide antigen, and wherein presentation is effected by administering, to the animal, a non-pathogenic microorganism or virus which is carrying at least one nucleic acid fragment encoding and expressing the first analogue.

51. The method according to any of claims 46-50, wherein the non-pathogenic microorganism is a bacterium.

52. The method according to claim 51, wherein the bacterium is selected from the genera *Escherichia*, *Bacillus*, *Salmonella*, and *Mycobacterium*.

53. The method according to claim 52, which is an *E. coli* cell or a *Mycobacterium bovis* BCG cell.

54. The method according to any of claims 46-50, wherein the non-pathogenic virus is a vaccinia virus strain.

55. The method according to any of claims 46-54, wherein the non-pathogenic microorganism or virus is administered once to the animal.

56. The method according to any one of claims 1-6, wherein presentation is effected by *in vivo* introducing, into the APC, at least one nucleic acid fragment which encodes and expresses

the at least one CTL epitope and/or the at least one B-cell epitope, and the at least one first foreign T_H epitope.

57. The method according to any one of claims 7-17, wherein presentation is effected by *in vivo* introducing, into the APC, at least one nucleic acid fragment encoding and expressing the first analogue.

58. The method according to any one of claims 18-30, wherein the T_H epitope and/or the first and/or second and/or third moieties are present in the form of fusion partners to the amino acid sequence derived from the cell-associated polypeptide antigen, and wherein presentation is effected by *in vivo* introducing, into the APC, at least one nucleic acid fragment encoding and expressing the first analogue.

59. The method according to any one of claims 13-17 and 57, which further comprises *in vivo* introduction, into the APC, of at least one nucleic acid fragment encoding and expressing the second analogue.

60. The method according to any one of claims 18-30 and 48, wherein the T_H epitope and/or the first and/or second and/or third moieties are present in the form of fusion partners to the amino acid sequence derived from the cell-associated polypeptide antigen, and which further comprises *in vivo* introduction, into the APC, of at least one nucleic acid fragment encoding and expressing the second analogue.

61. The method according to any one of claims 1-6, wherein presentation is effected by *in vivo* co-introducing, into the APC, at least two nucleic acid fragments, wherein one encodes and expresses the at least one CTL epitope and wherein another encodes and expresses the at least one first foreign T_H epitope,

and wherein the first foreign T_H epitope is as defined in any one of claims 1, 2 and 21-24.

62. The method according to any one of claims 46-61, wherein the nucleic acid fragment(s) introduced is/are selected from naked DNA, DNA formulated with charged or uncharged lipids, DNA formulated in liposomes, DNA included in a viral vector, DNA formulated with a transfection-facilitating protein or polypeptide, DNA formulated with a targeting protein or polypeptide, DNA formulated with a targeting carbohydrate, DNA formulated with Calcium precipitating agents, DNA coupled to an inert carrier molecule, and DNA formulated with an adjuvant.

63. The method according to claim 62, wherein the adjuvant is selected from the group consisting of the adjuvants defined in any one of claims 34-43.

64. The method according to any one of claims 46-63, wherein the mode of administration is as defined in claim 44 or 45.

65. A method for selection of an immunogenic analogue of a cell-associated polypeptide antigen which is weakly immunogenic or non-immunogenic in an animal, said immunogenic analogue being capable of inducing a CTL response in the animal against cells displaying an MHC Class I molecule bound to an epitope derived from the cell-associated polypeptide antigen, the method comprising

- identifying at least one subsequence of the amino acid sequence of the cell-associated polypeptide antigen which does not contain known or predicted CTL epitopes,
- preparing at least one putatively immunogenic analogue of the cell-associated polypeptide antigen by introducing, in the amino acid sequence of the cell-associated polypeptide antigen, at least one T_H epitope foreign to the animal in a

position within the at least one subsequence identified in step a), and

- selecting the/those analogues prepared in step b) which are verifiably capable of inducing a CTL response in the animal.

66. The method according to claim 65, wherein the subsequence identified in step a) further does not contain cysteine residues, or, alternatively, wherein the T_H epitope introduced in step b) does not substantially alter the pattern of cystein residues.

67. The method according to claim 65 or 66, wherein the subsequence identified in step a) further does not contain known or predicted glycosylation sites, or, alternatively, wherein the T_H epitope introduced in step b) does not substantially alter the glycosylation pattern.

68. The method according to any of claims 65-67, wherein the subsequence identified in step a) contributes significantly to a patophysiological effect exerted by the cell-associated polypeptide antigen, and wherein the introduction in step b) of the foreign T_H epitope reduces or abolishes said patophysiological effect.

69. The method according to any of claims 65-68, wherein the subsequence identified in step a) is homologous to an amino acid sequence of a different protein antigen of the animal, and wherein the introduction of the T_H epitope in step b) substantially removes the homology.

70. The method according to any of claims 55-59, wherein introduction in step b) of the foreign T_H epitope results in preservation of a substantial fraction of B-cell epitopes of the cell-associated polypeptide antigen.

71. The method according to claim 70, wherein the analogue has the overall tertiary structure of the cell-associated polypeptide antigen.

72. The method according to any of claims 55-71, wherein the preparation in step b) is accomplished by molecular biological means or by means of solid or liquid phase peptide synthesis.

73. A method for the preparation of cell producing an analogue of a cell-associated polypeptide antigen, the method comprising introducing, into a vector, a nucleic acid sequence encoding an analogue which has been selected according to the method of any of claims 55-72 and transforming a suitable host cell with the vector.

74. A method for the preparation of an analogue of a cell-associated polypeptide antigen, the method comprising culturing the cell obtained according to the method of claim 73 under conditions facilitating expression of the nucleic acid sequence encoding the cell-associated polypeptide antigen, and recovering the analogue from the culture supernatant or from the cells.

75. The method according to claim 74 which further comprises the step of purifying the recovered analogue and, optionally subjecting the purified product to artificial post-translational modifications such as refolding, treatment with enzymes, chemical modification, and conjugation.

76. Use of an analogue of a cell-associated polypeptide antigen which is weakly immunogenic or non-immunogenic in an animal, said analogue comprising

- an amino acid sequence containing a substantial fraction of known and predicted CTL epitopes of the cell-associated polypeptide antigen, and
- at least one T_H epitope which is foreign to the animal,

for the preparation of a pharmaceutical composition for down-regulating the cell-associated polypeptide antigen in the animal.

77. The use according to claim 76, wherein the analogue further has the overall tertiary structure of the cell-associated polypeptide antigen and/or lacks putatively pathophysiologi-related amino acid residues of the cell-associated polypeptide antigen.

78. The use according to claim 76 or 77, wherein the substantial fraction of known and predicted CTL epitopes in the amino acid sequence of the analogue are recognized by at least 90% of the MHC-I haplotypes recognizing all the known and predicted CTL epitopes in the cell-associated polypeptide antigen.

79. The use according to any of claims 76-78, wherein substantially all known CTL epitopes of the cell-associated polypeptide antigen are present in the analogue.

80. The method according to any of claims 1-65 or the use according to any of claims 66-69, wherein the weak cell-associated antigen is selected from the group consisting of
prostate-specific membrane antigen (PSM),
Her2,
fibroblast growth factor, e.g. FGF-8 such as FGF-8A and FGF-8b,
human chorionic gonadotropin (hCG)
BAGE,
beta-actinin,
Carcino Embryonic Antigen (CEA),
Cathepsins
CD33,
CDK-4,
E6,
E7,

EGFR,
EGP40 (KSA),
GAGE,
Gastrin-releasing peptide (bombesin),
GnTV,
GP1,
gp100 (melanoma-associated),
gp75 (melanoma-associated),
IGFR1,
K3,
MAGE such as MAGE1 and MAGE3,
MART,
Matrix Metalloproteinase such as MMP2, MMP3, MMP7, and MMP9,
Mucin, such as MUC-1, MUC-2, MUC-3, and MUC-4, which is aberrantly glycosylated,
MUM-1,
p15 (melanoma-associated),
PAI-1,
PDGF,
Plasminogen such as uPA,
RAGE,
TGF-α,
TRP-1/gp-75
TRP-2,
Tyrosinase, and
ZAG.

81. The method or use according to claim 80, wherein the cell-associated polypeptide antigen is PSM

82. The method or use according to claim 81, wherein the foreign T-cell epitope is introduced in a part of the PSM amino acid sequence defined by SEQ ID NO: 2 positions 16-52 and/or 87-108 and/or 210-230 and/or 269-289 and/or 298-324 and/or 442-465 and/or 488-514 and/or 598-630 and/or 643-662 and/or 672-699.

83. The method or use according to claim 81 or 82 used in the treatment or amelioration of prostate cancer.

84. The method or use according to claim 80, wherein the cell-associated polypeptide antigen is fibroblast growth factor 8b (FGF8b).

85. The method or use according to claim 84, where the foreign T-cell epitope is introduced in a part of the FGF8b amino acid sequence defined by SEQ ID NO: 6 positions 1-54 and/or 178-215 and/or 55-58 and/or 63-68 and/or 72-76 and/or 85-91 and/or 95-102 and/or 106-111 and/or 115-120 and/or 128-134 and/or 138-144 and/or 149-154 and/or 158-162 and/or 173-177, and wherein the introduction preferably does not substantially involve amino acids 26-45 and amino acids 186-215.

86. The method or use according to claim 84 or 85 used in the treatment or amelioration of cancer such as prostate cancer and breast cancer.

87. The method or use according to claim 80, wherein the cell-associated polypeptide antigen is Her-2.

88. The method or use according to claim 87, wherein the foreign T-cell epitope is introduced in a part of the Her-2 amino acid sequence defined by SEQ ID NO: 4 positions 61-75 and/or 105-119 and/or 151-165 and/or 212-226 and/or 252-266 and/or 327-341 and/or 371-385) and/or 467-481 and/or 581-595 and/or 72-86 and/or 146-160 and/or 221-235 and/or 257-271 and/or 387-401.

89. The method or use according to claim 87 or 88 used in the treatment or amelioration of breast cancer.

90. An analogue of human PSM which is immunogenic in humans, said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of PSM and including at least one foreign T_H epitope as defined in any of claims 21-24.

91. The analogue according to claim 90, wherein the at least one foreign T_H epitope is present as an insertion in the PSM amino acid sequence or as a substitution of part of the PSM amino acid sequence or as the result of deletion of part of the PSM amino acid sequence.

92. The analogue according to claim 91, wherein the foreign T_H epitope is introduced in the positions defined in claim 72.

93. An analogue of human Her2 which is immunogenic in humans, said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of Her2 and including at least one foreign T_H epitope as defined in any of claims 21-24.

94. The analogue according to claim 93, wherein the at least one foreign T_H epitope is present as an insertion in the Her2 amino acid sequence or as a substitution of part of the Her2 amino acid sequence or as the result of deletion of part of the Her2 amino acid sequence.

95. The analogue according to claim 94, wherein the foreign T_H epitope is introduced in the positions defined in claim 75.

96. An analogue of human/murine FGF8b which is immunogenic in humans, said analogue comprising a substantial part of all known and predicted CTL and B-cell epitopes of FGF8b and including at least one foreign T_H epitope as defined in any of claims 21-24.

97. The analogue according to claim 96, wherein the at least one foreign T_H epitope is present as an insertion in the FGF8b amino

acid sequence or as a substitution of part of the FGF8b amino acid sequence or as the result of deletion of part of the FGF8b amino acid sequence.

98. The analogue according to claim 97, wherein the foreign T_H epitope is introduced in the positions defined in claim 78.

99. An immunogenic composition which comprises, as an effective immunogenic agent the analogue according to any of claims 90-98 in admixture with a pharmaceutically and immunologically acceptable carrier, vehicle, diluent, or excipient, and optionally an adjuvant.

100. The immunogenic composition according to claim 99, wherein the adjuvant is as defined in any of claims 34-43

101. The analogue according to any of claims 90-97 or the composition according to claim 99 or 100 for use as a pharmaceutical.

102. A nucleic acid fragment which encodes an analogue according to any of claims 90-97.

103. A vector carrying the nucleic acid fragment according to claim 102.

104. The vector according to claim 103 which is capable of autonomous replication.

105. The vector according to claim 103 or 104 which is selected from the group consisting of a plasmid, a phage, a cosmid, a mini-chromosome, and a virus.

106. The vector according to any of claims 103-105, comprising, in the 5'-3' direction and in operable linkage, a promoter for

driving expression of the nucleic acid fragment according to claim 102, optionally a nucleic acid sequence encoding a leader peptide enabling secretion of or integration into the membrane of the polypeptide fragment, the nucleic acid fragment according to claim 102, and a nucleic acid sequence encoding a terminator.

107. The vector according to any of claims 103-106 which, when introduced into a host cell, is integrated in the host cell genome.

108. The vector according to any of claims 103-106 which, when introduced into a host cell, is not capable of being integrated in the host cell genome.

109. The vector according to any of claims 106-108, wherein the promoter drives expression in a eukaryotic cell.

110. The vector according to any of claims 106-109, wherein the promoter drives expression in a prokaryotic cell.

111. A transformed cell carrying the vector of any of claims 103-100.

112. The transformed cell according to claim 111 which is capable of replicating the nucleic acid fragment according to claim 102.

113. The transformed cell according to claim 102, which is a microorganism selected from a bacterium, a yeast, a protozoan, or a cell derived from a multicellular organism selected from a fungus, an insect cell, a plant cell, and an mammalian cell.

114. The transformed cell according to claim 113 which is a bacterium of the genus *Escherichia*, *Bacillus*, *Salmonella*, or *Mycobacterium*.

115. The transformed cell according to claim 114, which is an *E. coli* cell.

116. The transformed cell according to claim 115, which is a non-pathogenic *Mycobacterium* cell such as *M. bovis* BCG.

117. The transformed cell according to any of claims 111-116, which expresses the nucleic acid fragment according to claim 102.

118. The transformed cell according to claim 117, which secretes or carries on its surface, the analogue according to any of claims 90-97.

119. A composition for inducing production of antibodies against PSM, Her2 or FGF8b, the composition comprising

13) a nucleic acid fragment according to claim 102 or a vector according to any of claims 103-108, and

14) a pharmaceutically and immunologically acceptable diluent and/or vehicle and/or carrier and/or excipient and/or adjuvant.

120. The composition according to claim 119, wherein the nucleic acid fragment is formulated as defined in any of claims 33-43.

121. A stable cell line which carries the vector according to any of claims 103-110 and which expresses the nucleic acid fragment according to claim 102, and which optionally secretes or carries the analogue according to any of claims 90-97 on its surface.

122. A method for the preparation of the cell according to any of claims 111-118, the method comprising transforming a host cell with the nucleic acid fragment according to claim 102 or with the vector according to any of claims 103-110.

Fig. 1

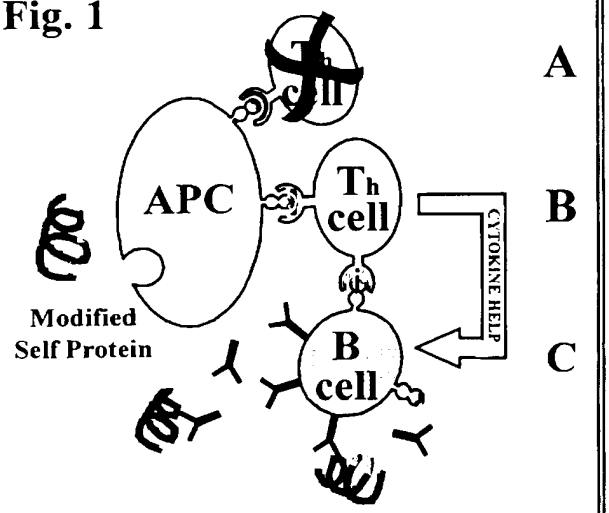
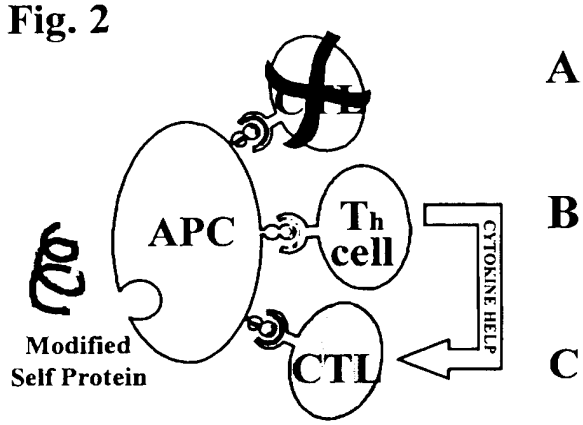


Fig. 2



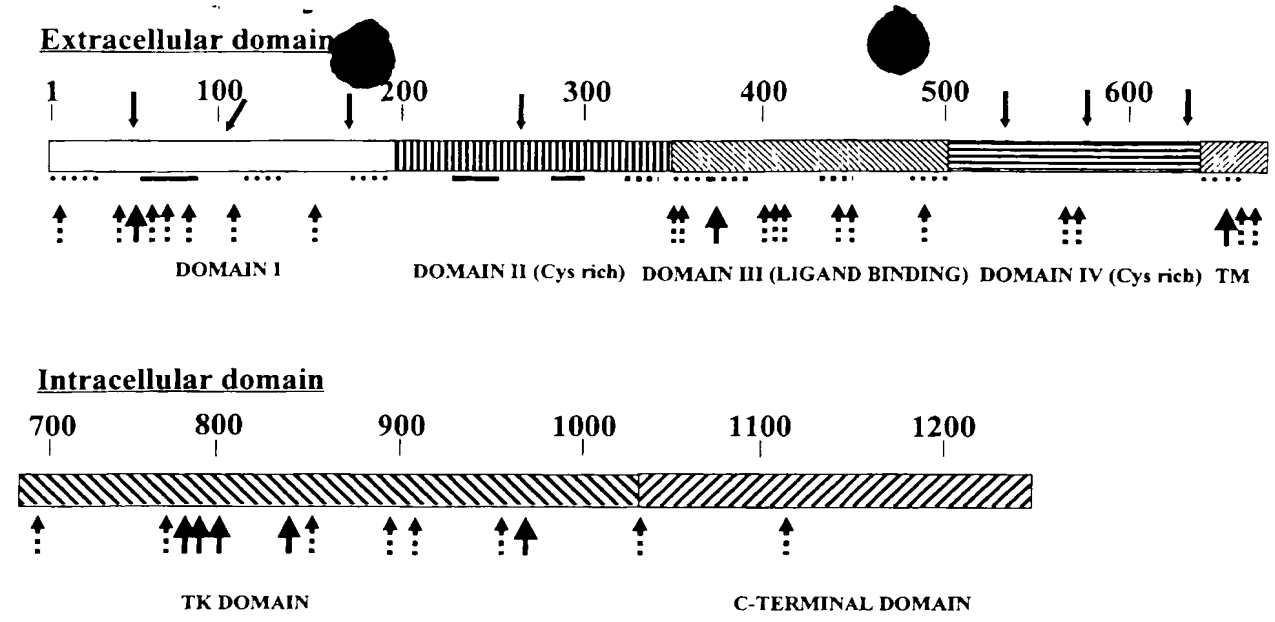


Fig. 3

Human PSM constructs

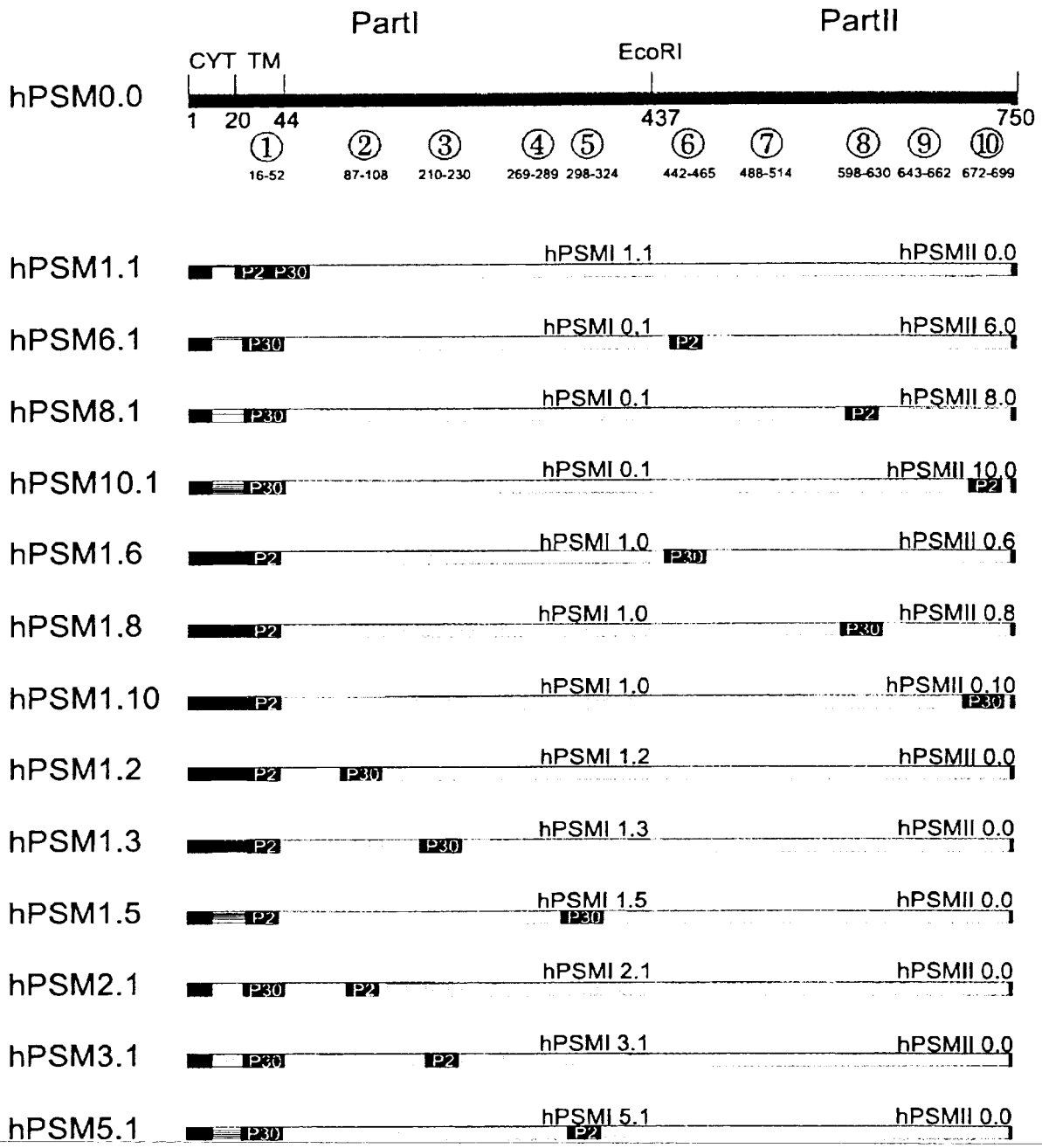


FIG. 4

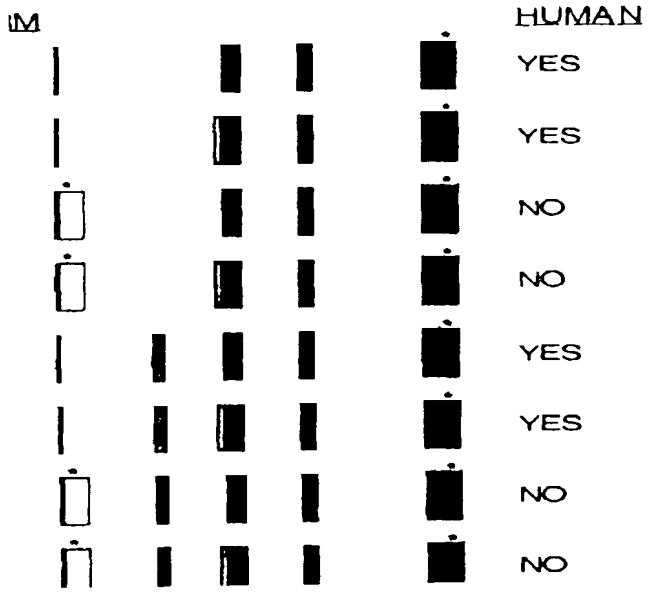
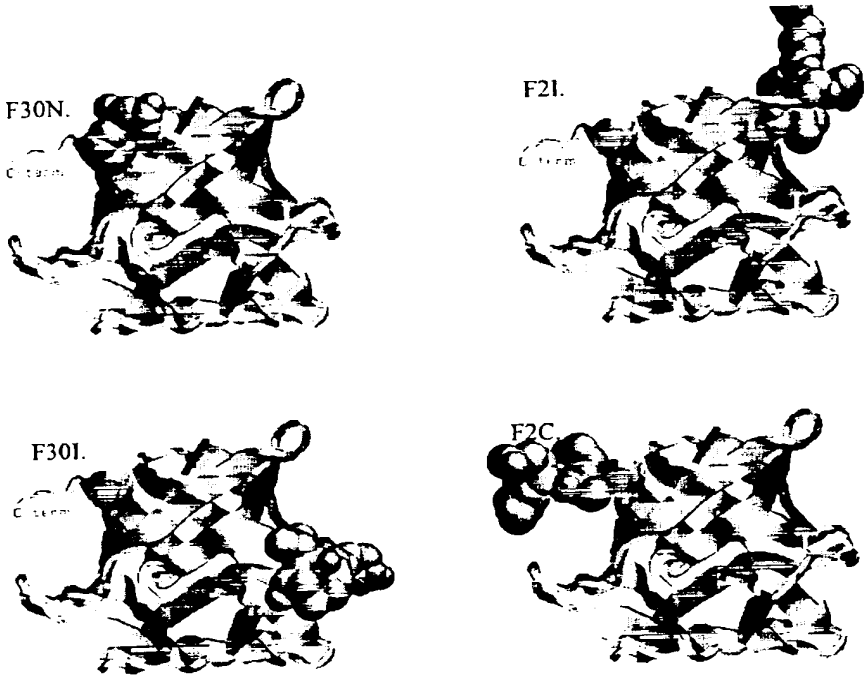


Fig. 5A

FGF8e and -f FGF8b and -f

MGSPRSALSC LLLHLLVLCL QA***QEGPGRGP ALGRELASLF RAGREPQGVS*** **QQVTVQSSPN** 31
FTQHVREQSL VTDQLSRRLI RTYQLYSRTS GKHVQVLANK RINAMAEDGD PFAKLIVETD 91
TFGSRVRVRG AETGLYI**C**MN KKGKLIAKSN GKGKD**C**VFTE IVLEN**N**YTAL QNAKYEGWYM 151
AFTRKGRPRK GSKTRQHQRE VHFMKRLPRG HHTTEQSLRF EFLNYPPFTR SLRGSQRTWA 211
PEPR 215

Fig. 5B

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F30N                         MAQVTVFNNFTVSFWLRVPKVSASHLERRLIRTYQLYSRTSGKHVQ  46
F2I                          MAQVTVQSSPNFTQHVREQSLVTDQLSRRLIRTYQLYSRTSGKHVQ  46
F30I                         MAQVTVQSSPNFTQHVREQSLVTDQLSRRLIRTYQLYSRTSGKHVQ  46
F2C                          MAQVTVQSSPNFTQHVREQSLVTDQLSRRLIRTYQLYSRTSGKHVQ  46

WT     VLANKRINAMAEDGDPFAKLIVETDTF                    GSRVRVRGAETGLYICMNKKGKLIAK 119
F30N   VLANKRINAMAEDGDPFAKLIVETDTF                    GSRVRVRGAETGLYICMNKKGKLIAK  99
F2I    VLANKRINAMAEDGDPFAKLIVETDQYIKANSKFIGITELGSRVRVRGAETGLYICMNKKGKLIAK 112
F30I   VLANKRINAMAEDGDPFAKLIVETDTF                    GSRVRVRGAETGLYICMNKKGKLIAK  99
F2C    VLANKRINAMAEDGDPFAKLIVETDTF                    GSRVRVRGAETGLYICMNKKGKLIAK  99

WT     SNG                        KGKDCVFTEIGLENNYTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 167
F30N   SNG                        KGKDCVFTEIGLENNYTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 147
F2I    SNG                        KGKDCVFTEIGLENNYTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 160
F30I   SNGFNNFTVSFWLRVPKVSASHLEDCVFTEIGLENNYTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 165
F2C    SNG                        KGKDCVFTEIGLENNYTALQNAKYEGWYMAFTRKGRPRKGSKTRQ 147


WT     HQREVHFMKRLPRGHHTTEQSLRFEFLNYPPFT  RSLRGSQRTWA  PEPR 215
F30N   HQREVHFMKRLPRGHHTTEQSLRFEFLNYPPFT  RSLRGSQRTWA  PEPR 195
F2I    HQREVHFMKRLPRGHHTTEQSLRFEFLNYPPFT  RSLRGSQRTWA  PEPR 208
F30I   HQREVHFMKRLPRGHHTTEQSLRFEFLNYPPFT  RSLRGSQRTWA  PEPR 213
F2C    HQREVHFMKRLPRGHHTTEQSLRFEFLNYPPFTQYIKANSKFIGITELPEPR 199
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Fig. 6